N- FERTILIZATION EFFECTS ON WATER- AND NITROGEN-USE EFFICIENCY IN Picea glauca FAMILIES

by

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ABSTRACT

Water-use efficiency (WUE) and nitrogen-use efficiency (NUE) are typically negatively correlated across environmental variation in N fertilization or drought. The contribution of genotypic and species NH₄⁺ preference in uptake to WUE and NUE was tested using ten full-sibling families of white spruce (Picea glauca (Moench) Voss). The families were raised hydroponically in solutions containing 100 μM N as NH₄⁺, NO₃⁻ or NH₄NO₃ and after eight weeks, growth parameters, gas exchange, C:N ratio and stable C and N isotope composition (δ¹³C and δ¹⁵N, respectively) were analyzed.

NH₄⁺ treatment significantly increased biomass which correlated negatively with NUE (C:N ratio). Although the significant difference in treatment mean biomass indicated a preference for NH₄⁺, there was no associated relationship with NUE or δ¹³C, and a negative correlation between NUE and WUE (as measured by gas exchange and δ¹³C) did not occur across treatments. Potential reasons for the lack of correlation are the significant variation between treatment replicates or an unknown effect of hydroponics on gas exchange in this species. In contrast to treatment effects, families were significantly different in biomass, C:N ratio and δ¹³C, and each family maintained its rank in the measured parameters. This indicated that there are high uptake and low uptake families regardless of the form of nitrogen supplied. There was no evidence of a genotypic trade-off between WUE and NUE presumably because of the interaction of genetic control of N assimilation and allocation to growth versus non-growth related N compounds.

Mean Δδ¹⁵N was significantly different in each of the treatments and positively correlated with biomass. A potential explanation for treatment differences relates to the efflux/influx ratio associated with each of the N forms. Slower uptake and assimilation of NO₃⁻ over NH₄⁺ combined with a low storage capacity, could cause greater efflux of ¹⁵N yielding more negative Δδ¹⁵N values in the NO₃⁻ treatment than in the NH₄⁺ and NH₄NO₃ treatments. Stable N isotope composition was also shown to be genetically controlled and correlated positively with δ¹³C. This correlation may be a result of genetically determined uptake rates and sink strength, such that if a family has a high uptake rate and allocates a greater proportion of assimilated N to Rubisco, this will yield less negative δ¹³C and Δδ¹⁵N values.
A second experiment was designed to test the physiological control of N isotope fractionation. The experimental design was similar to the first experiments but the treatments imposed were steady-state and draw-down supply rates of 200 μM NH₄⁺. There was no significant difference between treatments in total biomass, but the ratio of root dry matter to shoot dry matter was higher in the draw-down treatment indicating a higher level of nutrient stress. Treatments also significantly affected C:N ratio, δ¹³C and C/Cₐ, resulting in a positive environmental correlation between NUE and δ¹³C.

Treatment means for Δδ¹⁵N were not significantly different possibly because the ¹⁵N enrichment of the medium overcame enzymatic discrimination. Genotypic control of Δδ¹⁵N was significant and may reflect genetic control of the balance of N efflux/influx at the roots. This balance of efflux/influx may be related to genetic control of N uptake, assimilation, allocation and demand.
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<table>
<thead>
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<tbody>
<tr>
<td>$A$</td>
<td>assimilation rate ($\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$)</td>
</tr>
<tr>
<td>$A_{\text{max}}$</td>
<td>assimilation capacity ($\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$)</td>
</tr>
<tr>
<td>Arg</td>
<td>arginine</td>
</tr>
<tr>
<td>AS</td>
<td>asparagine synthetase</td>
</tr>
<tr>
<td>Asn</td>
<td>asparagine</td>
</tr>
<tr>
<td>Asp</td>
<td>aspartate</td>
</tr>
<tr>
<td>$\text{Ci}/\text{C}_a$</td>
<td>internal CO$_2$ concentration/ambient CO$_2$ concentration</td>
</tr>
<tr>
<td>CHATS</td>
<td>constitutive high affinity transport system</td>
</tr>
<tr>
<td>$\delta^{13}\text{C}$</td>
<td>stable C isotopic composition (‰)</td>
</tr>
<tr>
<td>$\delta^{15}\text{N}$</td>
<td>stable N isotopic composition (‰)</td>
</tr>
<tr>
<td>$\Delta$</td>
<td>isotopic discrimination (‰)</td>
</tr>
<tr>
<td>$\Delta\delta^{15}\text{N}$</td>
<td>stable N isotopic composition of the plant minus the salt (‰)</td>
</tr>
<tr>
<td>$E$</td>
<td>transpiration rate ($\mu$mol H$_2$O m$^{-2}$ s$^{-1}$)</td>
</tr>
<tr>
<td>GDH</td>
<td>glutamate dehydrogenase</td>
</tr>
<tr>
<td>Gln</td>
<td>glutamine</td>
</tr>
<tr>
<td>Glu</td>
<td>glutamate</td>
</tr>
<tr>
<td>GOGAT</td>
<td>glutamate synthase</td>
</tr>
<tr>
<td>GS</td>
<td>glutamine synthetase</td>
</tr>
<tr>
<td>GS2</td>
<td>chloroplastic glutamine synthetase</td>
</tr>
<tr>
<td>$g_s$</td>
<td>stomatal conductance ($\mu$mol H$_2$O m$^{-2}$ s$^{-1}$)</td>
</tr>
<tr>
<td>g x e</td>
<td>genotype x environment interaction effect</td>
</tr>
<tr>
<td>HATS</td>
<td>high affinity transport system</td>
</tr>
<tr>
<td>$K_m$</td>
<td>external substrate concentration at $\frac{1}{2} V_{\text{max}}$ (μM)</td>
</tr>
<tr>
<td>LATS</td>
<td>low affinity transport system</td>
</tr>
<tr>
<td>NiR</td>
<td>nitrite reductase</td>
</tr>
<tr>
<td>$N_{\text{leaf}}$</td>
<td>N concentration of the leaf</td>
</tr>
<tr>
<td>$[\text{NO}_3^-]_o$</td>
<td>external NO$_3^-$ concentration</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NR</td>
<td>nitrate reductase</td>
</tr>
<tr>
<td>NUE</td>
<td>nitrogen-use efficiency</td>
</tr>
<tr>
<td>PEPcase</td>
<td>phosphoenolpyruvate carboxylase</td>
</tr>
<tr>
<td>PNUE</td>
<td>photosynthetic nitrogen-use efficiency</td>
</tr>
<tr>
<td>PON</td>
<td>particulate organic nitrogen</td>
</tr>
<tr>
<td>R:S ratio</td>
<td>ratio of root dry mass to shoot dry mass</td>
</tr>
<tr>
<td>RuBP</td>
<td>ribulose bisphosphate</td>
</tr>
<tr>
<td>SLM</td>
<td>specific leaf mass (g m$^{-2}$)</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>maximal transport when all carrier sites are loaded ($\mu$mol g$^{-1}$ h$^{-1}$)</td>
</tr>
<tr>
<td>vpd</td>
<td>vapor pressure deficit (mbar)</td>
</tr>
<tr>
<td>WUE</td>
<td>water-use efficiency</td>
</tr>
<tr>
<td>WUE$_i$</td>
<td>instantaneous water-use efficiency</td>
</tr>
<tr>
<td>%‰</td>
<td>per mil, parts per thousand</td>
</tr>
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</table>
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INTRODUCTION

One of the determinants of competitive success in plant communities is how species and individuals utilize available resources to achieve maximal growth. Most important to competitive success is optimizing the use of the most limiting resource. The concept of plant strategies to increase the use efficiency of the most limiting resource was first introduced by Chapin (1980) who used the common definition of resource use efficiency as the amount of biomass produced per unit of resource. For example, water-use efficiency (WUE) is typically defined as the amount of dry matter yield for water transpired. This balance between transpiration and carbon gain depends on stomatal conductance ($g_s$) and assimilation capacity ($A_{max}$) which can both be measured using gas exchange equipment to provide an instantaneous measure of WUE (Farquhar and Sharkey, 1982). The ratio of stable isotopes of carbon has also been theoretically and empirically determined to be a valid indicator of WUE on a long-term scale (Farquhar et al., 1982; Evans et al., 1986; Meinzer et al., 1992; O'Leary, 1993). Nitrogen-use efficiency (NUE) is measured as gram of carbon per gram of nitrogen in the plant. Similarly, short-term NUE is defined as the assimilation capacity of CO$_2$ per gram of leaf nitrogen ($A_{max}$/N$_{lean}$) (Reich and Walters, 1994).

Theoretically, the efficient use of water and nitrogen should trade-off on an environmental basis. Under irrigated conditions, plants can optimize NUE by increasing $g_s$ and, consequently, photosynthesis and the uptake of mineral nutrients (Schulze and Ehleringer, 1984). During periods of drought, stomatal closure increases WUE and, as a result of decreased carbon assimilation, NUE decreases (Sheriff et al., 1986; Reich et al., 1989; Livingston et al., 1999). This trade-off is also evident on a species basis, such that plants which have adapted to droughty environments optimize the efficient use of water at the expense of nitrogen (N) (Field et al., 1983; DeLucia and Schlesinger, 1991; Patterson et al., 1997). Whether or not there is a similar trade-off at the genotypic level within species has yet to be determined. If so, then genetic differences in WUE may, at least in part, be driven by genetic differences in N acquisition.

Ammonium (NH$_4^+$) preference in uptake, as compared to nitrate (NO$_3^-$), increases the N-content of conifers (Sheriff et al., 1986; Scheromm and Plissant, 1988; Marschner et al., 1991; Lavoie et al., 1992; Kronzucker et al., 1995; Kronzucker et al., 1996; Korol et al., 1999). Because approximately 75% of internal N can be allocated to photosynthetic machinery (Field
and Mooney, 1986), there is a tight correlation between the N-quantity of a leaf and CO₂ assimilation (Sage and Pearcy, 1987; Evans, 1989; Reich et al., 1995). Therefore, treatments of NH₄⁺ versus NO₃⁻ should expose the environmental trade-off between NUE and WUE through differences in N-quantity of the leaf.

Although gross differences in species N-source preferences have been shown, more subtle genotypic preferences through differing quantities or affinities of uptake carriers or assimilatory enzymes have not. If there is genetic control of N-source preference, then relative performance of particular genotypes may be different on NO₃⁻ as compared to NH₄⁺. This could change the WUE and NUE rank of the genotypes between treatments. If the quantity and affinity of uptake carriers are somehow linked, either genetically or physiologically, then genotypes will maintain WUE and NUE rank in all treatments providing no additional information. If however, ranks change and WUE and NUE remain negatively correlated, this pattern would provide strong support for the notion that the ability to acquire N underlies genetic differences in WUE. Finally, WUE rank might change independently of N-source, suggesting that ability to acquire N is not a primary determinant of WUE.

Unlike fractionation of carbon isotopes in terrestrial plants, the physiology behind the fractionation of N stable isotopes, ¹⁵N and ¹⁴N, is still unclear. For the most part, the ratio of stable isotopes has been used for labeling purposes in ecology to investigate N-partitioning within plants and soil (Buchmann et al., 1996; Stark and Hart, 1997; Nasholm et al., 1998; George et al., 1999) or competition between differing species (Staples et al., 1999). In terrestrial systems, natural abundance levels have most frequently been used to discern between fertilization effects (Johannisson and Hogberg, 1994; Hogberg et al., 1995; Neilson et al., 1998; Schulze et al., 1998; Schulze et al., 1999) or diazotrophy (Shearer and Kohl, 1989; Handley and Raven, 1992; Roggy et al., 1999). In oceanography, natural variation in ¹⁵N has been used extensively in food web studies and is under investigation in the context of the physiology of N-uptake by phytoplankton. A major goal is to apply an understanding of N isotope fractionation to analyzing sediment layers for information about past productivity (Hoch et al., 1992; Waser et al., 1998; Waser et al., 1998; Waser et al., 1999).

Correlations between C isotope fractionation in photosynthesis and N fractionation during uptake and assimilation were first proposed by Mariotti et al. (1981) using soil bacteria. They suggested that in an open system, where N can diffuse into and out of the cell,
discrimination should be high. When this exchange is terminated through exhaustion of external nitrogen or when N assimilation rate equals gross uptake, there will be no fractionation against $^{15}$N (Mariotti et al., 1981). Discrimination by isolated enzymes (Ledgard et al., 1985), and the $\delta^{15}$N values of isolated amino acids (Shearer et al., 1989) and plant parts (Handley et al., 1997a; Handley et al., 1997b) have all been determined experimentally. Combined, these pieces of information contribute to our understanding of the mechanics of discrimination within the plant, but on their own do not account for total plant discrimination. Whole plant discrimination must incorporate some type of N-loss through efflux of inorganic N prior to assimilation, exudation of organic N, gaseous NH$_3$ loss, or selective senescence of isotopically different organs. The majority of research points to the first explanation as the most likely, although the controls of efflux are still unknown. Plant efflux of $^{15}$N enriched inorganic N can depend on multiple factors related to both the form of N-fertilization and plant N-status. Differences in uptake, storage and assimilation capacities related to the form of N supplied may alter discrimination through inorganic N efflux. There may also be discrimination inherent in the assimilating enzymes or the equilibrium isotope effect associated with NH$_3$-NH$_4^+$ reduction. As well, the form of N may affect internal N-status, thereby altering efflux and exudation of heavy N. Apart from these effects particularly related to N form, plant N-status could also affect the amount of inorganic N effluxed as well as organic N exudates.

This study was designed to elucidate the effects of nitrogen source on the genetic control of water-use efficiency in white spruce (Picea glauca (Moench) Voss) seedlings. In addition, the use of $\delta^{15}$N in studies on nitrogen relations was investigated. The following hypotheses were considered:

Hypotheses

1. Based on a preference in uptake, NH$_4^+$ fertilization will increase the WUE of white spruce by providing more N for photosynthetic proteins.
2. Within each treatment, there will be genetic control of WUE and NUE.
3. There will be genotypic variation in NH$_4^+$ and NO$_3^-$ uptake capacity which will alter family WUE and NUE ranking between treatments.
4. This alteration of rank will modify the environmental trade-off between WUE and NUE.
5. There will be an N-source treatment effect on plant $\delta^{15}\text{N}$ reflecting differences in N uptake and assimilation.

6. There will be an N-supply treatment effect on plant $\delta^{15}\text{N}$ dependent on internal N status.

7. Genetic control of plant $\delta^{15}\text{N}$ will be shown by significant differences between genotypes.

8. Genotypes will not change their $\delta^{15}\text{N}$ rank across treatments, thereby indicating that genetic control of discrimination is consistent in varying environments.
LITERATURE REVIEW

I. Water-use Efficiency (WUE)

A. Physiology and Measurement of WUE

The water-use efficiency (WUE) of a plant or crop is generally defined as the amount of dry mass gained through carbon assimilation \( A \) for the amount of water lost in transpiration \( E \). This ratio varies between life forms (DeLucia et al., 1991; Brooks et al., 1997) and habitats (Field et al., 1983; Meinzer et al., 1992); it is influenced by both environmental (Yoshie, 1986; Reich et al., 1989) and genetic factors (Virgona et al., 1990; Zhang and Marshall, 1994); and it can have varying effects on the competitive success of a species or a genotype (DeLucia and Heckathorn, 1989; Patterson et al., 1997). Research devoted to understanding this balance is plentiful and has provided insight on photosynthesis, stomatal conductance, carbon allocation, drought tolerance and nutrient stress.

WUE can be measured and expressed in different ways. Long term WUE is measured as the ratio of dry matter production to water taken up by the plant over a particular time period (Kramer, 1983). This is often done using a lysimeter or before and after weight measurements. Instantaneous WUE \((WUE_{i})\) is often measured as the ratio \( A/E \) using gas exchange equipment. This ratio is dependent on the respective diffusion gradients for \( \text{CO}_2 \) and water:

\[
WUE_{i} = \frac{A}{E} = \frac{g_c (C_a - C_i)}{g_w (e_a - e_i)} = \rho_a \left( 1 - \frac{C_i}{C_a} \right) 1.6 \frac{v}{v}
\]

where \( C_a \) and \( C_i \) are the ambient and intercellular concentrations of carbon dioxide, \( g_c \) and \( g_w \) are the conductances to the diffusion of \( \text{CO}_2 \) and water vapor, \( e_a \) and \( e_i \) are the atmospheric and intercellular vapor pressures and \( v \) is the vapor pressure difference between them. The difference between vapor pressures is multiplied by 1.6 to account for the greater conductance of water than \( \text{CO}_2 \) in air (Farquhar et al., 1989).

Where \( v \) is held constant or is comparable between plants or across treatments, variation in \( WUE_{i} \) is determined by variation in \( C_i/C_a \). The ratio of \( \text{CO}_2 \) concentrations is dependent on both stomatal conductance \( (g_s) \) and photosynthetic capacity \( (A_{\text{max}}) \). The ratio will increase if either \( g_s \) decreases and \( A_{\text{max}} \) remains unchanged or if \( A_{\text{max}} \) increases while \( g_s \) remains constant. In either situation, low \( C_i \) is caused by stomatal limitation of \( A \) which yields a high WUE. At high \( C_i \), \( A \) continues to be partially limited by \( g_s \), but becomes more limited by the quantity of
ribulose bisphosphate (RuBP). Regeneration of RuBP depends on electron transport rate which is in turn, dependent on illumination (Farquhar et al., 1982). Research has shown that stomata generally operate to maintain a photosynthetic rate that exists between being primarily limited by $g_s$ or being limited by $g_s$ and RuBP regeneration. It is at this point that the plant has optimized water-use efficiency (von Caemmerer and Farquhar, 1981).

A second long-term measure of WUE that relates specifically to $C_i/C_a$ is the ratio of stable carbon isotopes in the plant. Atmospheric CO$_2$ is primarily comprised of two stable isotopes, $^{12}$C and $^{13}$C, in the ratio 98.9:1.1. As CO$_2$ diffuses through the plant cell and is assimilated by carboxylating enzymes, this ratio within the plant tissue is altered slightly in favor of the lighter isotope $^{12}$C. Both diffusion and assimilation discriminate against the heavier isotope which may flow back out of the stomatal pore. The amount of discrimination varies with $C_i/C_a$ and the inherent discrimination of the carboxylating enzyme. Net discrimination against CO$_2$ is maximal when diffusion through the stomata permits constant and full renewal of the CO$_2$ in the intercellular leaf spaces (i.e., when $C_i$ is equal to $C_a$). Fractionation is minimal when diffusion is unidirectional and fully limiting to photosynthesis. Net discrimination is normally between these two extremes as a function of the balance between $g_s$ and $A_{\text{max}}$. The ratio of the two isotopes is most often written as the isotopic composition, $\delta^{13}$C, of air or the plant as compared to the standard, PDB. PDB was a fossil belemnite from the Pee Dee formation. Other standards, referenced to PDB by international convention (“Vienna PDB”) are used in practice. The equation for expressing isotopic composition relative to a standard is:

$$\delta^{13}\text{C} \%o = \frac{^{13}\text{C}/^{12}\text{C}_{\text{sample}}}{^{13}\text{C}/^{12}\text{C}_{\text{standard}}} x 1000 \tag{2}$$

Because PDB is calcium carbonate of marine origin, most $\delta^{13}$C values referenced to it are negative. The isotopic composition of the air is approximately $-8\%o$ and for C3 plants is between $-22\%o$ and $-34\%o$ depending on species and environmental conditions. The more negative the value, the smaller the ratio of $^{13}$C/$^{12}$C in the sample and the greater the discrimination. (Farquhar et al., 1989b)

Discrimination, relative to a source (e.g. air), is described in terms of $\Delta$ values, which are positive. It can be calculated from $\delta^{13}$C values according to the following expression:

$$\Delta = \frac{\delta_a - \delta_p}{1 + \delta_p} \tag{3}$$
where $\delta_a$ is the $\delta^{13}C$ of air and $\delta_p$ is the $\delta^{13}C$ of the plant. The benefit of using $\Delta$ is that it can be directly related to the additive effects of diffusion and carboxylation weighted by $C_i$.

$$\Delta = a + (b - a)\frac{C_i}{C_a}$$  \hspace{1cm} (4)

In equation (4), $a$ is the discrimination that occurs naturally in air by diffusion (4.4%) and $b$ is the discrimination occurring through the combined carboxylations by both Rubisco and PEP carboxylase (PEPcase) (27%). Rubisco and PEPcase independently have very different $\Delta$ values, whereby Rubisco discriminates more heavily than PEPcase ($\Delta = 29\%$ vs. 2%). As the percentage of carbon assimilation by PEPcase increases, plant discrimination decreases. (O'Leary, 1993)

Since the discovery of the relationship between $C_i/C_a$ and $\delta^{13}C$, stable isotopes have been used repeatedly to indicate differences between photosynthetic pathways, stress tolerance and species and genotype differences in WUE. When used appropriately, plant stable isotopic composition can provide a more integrated, long-term and precise index of WUE than gas exchange because the ratio is based on the entire plant and incorporates life-time WUE. For example, $\delta^{13}C$ values have been used to show variations in WUE related to species and habitat associated differences in $C_i$. Brooks et al. (1997) surveyed different life forms (trees, shrubs, forbs, and mosses; deciduous or evergreen) and found that life form described a significant portion of the variation in WUE. The correlations between life form and WUE were related to plant stature and leaf longevity. When comparing deciduous and evergreen forbs, shrubs and trees, shrub species were most alike one another. Brooks et al. (1997) attributed the difference in forbs to variation in altitudinal gradient, and in trees to the closer coupling with the environment in evergreen species. They proposed that within the plant's native environment, each life form operates with the optimal WUE.

Environmental control of WUE may be tested by manipulating the environment and describing the plastic response of a plant. Common environmental manipulations are water and nitrogen stress. Mild to moderate water stress primarily results in partial stomatal closure. This usually causes a proportionally greater decrease in $E$ than $A$, such that $C_i$ goes down and WUE increases (Farquhar et al., 1982). Nitrogen fertilization primarily affects WUE by increasing the quantity of photosynthetic proteins. If a chloroplast is composed of 25–33% stromal protein, of which one half is in carboxylase, any increase in the N content of the leaf ($N_{leaf}$) significantly
affects $A$ by increasing the quantities of Rubisco and RuBP (von Caemmerer and Farquhar, 1981). A correlation between photosynthetic rate and $N_{leaf}$ has been substantiated in multiple publications (Field et al., 1986; Evans, 1989; Reich et al., 1995). Although N fertilization may increase $A_{max}$, there can be two effects on WUE depending on $g_s$. If $g_s$ is constant, an increase in $A_{max}$ will cause a decrease in $C_i$. On the other hand, if the increase in $A_{max}$ is accompanied by a relatively smaller increase in $g_s$ then $C_i$ will remain constant, thereby maintaining WUE. Wong et al. (1979) did a series of experiments designed to perturb either $A$ or $g_s$ of three different species of plants. They found that when $A$ changed, $g_s$ was modified and a constant $C_i$ was maintained. One of the treatments imposed was four levels of nitrogen nutrition (Wong et al., 1979).

On a whole plant scale, water and nitrogen stress can affect WUE through alterations in biomass allocation or relative growth rate. Nitrogen and water stress both increase the ratio of root dry mass to shoot dry mass (R:S ratio) (Hubick, 1990; Patterson et al., 1997). This can have varying effects on both long- and short-term WUE and their relationship with biomass dependent on the type of stress. Under water stress, generally there is a negative correlation between the two traits because of induced stomatal closure. Between N levels, there is typically a positive relationship, reflecting a decrease in $A_{max}$ (Virgona and Farquhar, 1996).

Although a high WUE may seem like a positive trait, it can also decrease the plants efficient use of N for photosynthesis and can allow available soil water to be lost to direct evaporation and competitors. When these two factors are considered, a high WUE may not always be the best strategy for survival. For example, black spruce may trade-off efficient use of water for more efficient use of N because in its indigenous habitat of nutrient poor muskegs, water is plentiful but N is not (Patterson et al., 1997). In desert soils, a plant may exhibit a high drought tolerance, but a low WUE in order to out-compete its neighbors for water (DeLucia et al., 1991). Conversely, WUE may increase during drought by a reversible change in metabolism from C3 to CAM photosynthesis (O'Leary, 1993). Studying available nitrogen levels in the soil and interactions with competitors is important in assessing the ecophysiological benefits of the inherent WUE of a species.
B. Genetic control of WUE

Condon and Richards (1992) define broad sense heritability as “the proportion of total phenotypic variance that is attributable to genotypic differences”. If genotypes maintain their relative WUE ranking through different environmental conditions, then there is little genotype x environment interaction (g x e interaction) and WUE is a more reliable criterion for selection. If the g x e interaction is high, there could still be a high heritability within a given environment because the genotype response to that environment is consistent, but WUE may no longer be a reliable criterion for predicting performance across environments. If gas exchange or isotopic measures of WUE are to be used for selection, then the heritability of these two traits must parallel that of WUE.

Most research on genotypic variation in WUE has used crop plants, with differing results for heritability (Farquhar and Richards, 1984; Hubick et al., 1988; Condon et al., 1990; Virgona et al., 1990; Condon et al., 1992). In wheat, Farquhar and Richards (1984) showed that isotopic and gas exchange measures of WUE correlated in well-watered plants. Variation in WUE was attributed approximately equally to variations in leaf conductance and $A_{\text{max}}$ when plants were well fertilized but subjected to varying amounts of water stress (Condon et al., 1990). Broad sense heritability for isotope discrimination, $\Delta$, was often greater than 90% and discrimination changed substantially as a result of both genetics and environment but the two factors did not interact (Condon et al., 1992). The authors concluded that the high heritability estimates and lack of a g x e interaction permit $\Delta$ to be used as an effective selection trait under well-watered conditions. Similarly, for peanut, there was no g x e interaction when plants were subjected to drought stress or well-watered environments (Hubick et al., 1988). Broad sense heritability was 81% in this case.

Fewer studies have been done on relationships between gas exchange parameters, isotope discrimination and WUE in gymnosperms. Zhang and Marshall (1994) demonstrated population differences in WUE of western larch (Larix occidentalis) seedlings using $\Delta$ values, but did not find significant differences in WUE$_i$ using gas exchange. The seedling WUE rank by $\Delta$ values was maintained in both well-watered and water-stressed soils, indicating no g x e interaction, and it correlated well with altitudinal differences in seed source. The lack of variation in WUE$_i$ was attributed to low variability in physiological characteristics in western larch families, environmental fluctuations which can affect the WUE$_i$ of the plant and a small number of
replications within populations (Zhang and Marshall, 1994). In a later paper (Zhang et al., 1994), the same parameters were measured on twelve-year old seedlings from different western larch families. In this case there were large fluctuations in $A$, $E$ and $g_s$ over the growing season which may have prevented a visible difference in $WUE_i$ between genotypes. Although there was no detectable difference between families in $WUE_i$, there was a strong negative correlation with $\Delta$ values of the families. $\Delta$ also correlated with growth suggesting that genotypic differences in $WUE$ were related to $A_{\text{max}}$.

Holowachuk (1993) compared $\delta^{13}C$ values of 11 lodgepole pine provenances (ten $Pinus contorta$ var. *latifolia* and one var. *contorta*) from various climates in British Columbia. The trees were growing in provenance trials at three sites varying in water supply. Across the sites, the trees maintained their WUE ranking which roughly correlated with precipitation levels from their site of origin. The only anomaly was a population (var. *contorta*) from the coastal region. This region is typically wetter than any of the other provenances, yet this population had a higher WUE both in the field trials and the greenhouse study. She suggested this was not because of higher $A_{\text{max}}$, but perhaps due to heightened stomatal sensitivity to vapor pressure deficit (vpd) (Holowachuk, 1993).

In two studies using ten controlled crosses of white spruce ($Picea glauca$), genetic control of WUE and a correlation between gas exchange and stable isotope composition was shown (Sun et al., 1996; Livingston et al., 1999). In the first study, plants maintained their stable isotope ranking across water treatments and WUE (measured by biomass and time domain reflectometry of soil moisture) corresponded with $WUE_i$ and with stable isotope values. All three variables were correlated through gas exchange differences and not morphology. Both $\delta^{13}C$ and biomass measures of WUE were positively correlated with dry matter production. This result, combined with a lack of g x e interaction effect, provided evidence that genetic differences in WUE were attributable to $A_{\text{max}}$. A positive correlation between $\delta^{13}C$ and growth has also been shown in peanut (Hubick, 1990), sunflower (Virgona et al., 1990) and larch (Zhang et al., 1994).

The second study (Livingston et al., 1999) included a N stress component intended to alter $A_{\text{max}}$. Again there was no significant g x e interaction, WUE indices correlated across all treatments, there was a significant genetic effect on each index, and biomass was positively correlated with WUE and $\delta^{13}C$. One interesting point is that when $A_{\text{max}}$ increased with
fertilization, the genetic differences in WUE under droughted conditions tended to become more apparent. Although the genetic differences are determined by $A_{\text{max}}$, water limitations on $E$ may enhance the effect of $A_{\text{max}}$ on WUE. This is one of very few studies in the literature that has looked at the genetic component of the interaction between WUE and NUE.

II. Nitrogen-use Efficiency (NUE)

A. Physiology and Measurement of NUE

Nitrogen-use efficiency (NUE) can be measured on both short- and long-term bases. Instantaneous NUE or photosynthetic NUE (PNUE) is measured as $A_{\text{max}}$ per unit leaf N ($A_{\text{max}}/N_{\text{leaf}}$) (Reich et al., 1989). This measure does not include N in the branches, stems or roots, nor does it differentiate between leaf retention and retranslocation which can vary in both deciduous and evergreen trees. It is often used similarly to WUE$_i$ to indicate the potential NUE of a species under optimal conditions. A high $N_{\text{leaf}}$ can be associated with lower C assimilation per unit N, so PNUE decreases. Gas exchange measurements for PNUE are usually done over short intervals giving an instantaneous index of NUE (DeLucia and Schlesinger, 1995).

Long-term NUE is generally measured as C/N ratio. When measured on a whole plant basis, NUE incorporates allocation of N to different plant parts. Allocation can refer to the proportion of N devoted to Rubisco, structural proteins, defense compounds, N stored in the vacuole or luxury consumption. Allocation can also refer to how C is distributed in the plant, for example N nutrition can affect the thickness of a leaf or specific leaf mass (SLM) and R:S ratio. A high N supply can increase the leaf surface area over the plant providing more C gain per unit N (Field and Mooney, 1986). Walters and Reich (1989) found this to be true with *Ulmus americana*; under high N supply the leaves produced a greater surface area with a higher N content and a greater leaf C gain (Walters and Reich, 1989). R:S ratio generally decreases under conditions of high N supply (Chapin, 1980; Birk and Vitousek, 1986) and is commensurate with a low C:N because of the proportionally higher quantities of N in the shoot than the root.

Long-term NUE is also used in studies on forest production, where it again incorporates even more facets and as a result is often harder to measure. First is the logistical problem of analyzing the NUE of entire trees. It has been proposed to use the N content of litterfall as an index of NUE based on the presumption that trees which grow on low-N sites will be more efficient, have higher rates of retranslocation and will therefore have a low $N_{\text{leaf}}$ in the litterfall.
(Vitousek, 1982). Later research has shown that this difference in NUE is not a plastic change in retranslocation related to site fertility, but rather a result of sink strength (i.e. shoot growth rate). Growth rate may be correlated with nutrient availability, but it can also be related to age, seasonality in growth patterns and longevity of leaves (Birk et al., 1986; Nambiar and Fife, 1991). A second problem associated with measuring NUE in the forest is the influence of mycorrhizae on uptake. Mycorrhizae present in the soil have been shown to affect uptake either by buffering the pH changes caused by ion uptake or by increasing the uptake of differing forms of mineral N (Rygiewicz et al., 1984; Rygiewicz et al., 1984). The associations between particular tree species and fungi then could influence the N content of specific trees in a community.

Although understanding NUE both on an instantaneous and long-term basis is helpful, this literature review will continue by focusing only on the environmental and genetic components of whole plant NUE. The treatment effects of differing N sources on uptake, assimilation and allocation capacities are all incorporated in the use of C/N ratio. Uptake capacity for particular nitrogen forms could influence the NUE by providing more or less N to the plant. Furthermore, assimilation and allocation differences can determine whether the nitrogen that is taken up is used for carbon assimilation (helping to maintain NUE) or storage (decreasing NUE).

1. Kinetics of nitrogen uptake

Environmentally induced changes in uptake rate at low concentrations are caused by both a preference for one N-form over another and pre-treatment N concentrations. In one of the first papers on N uptake, Clement et al. (1978) suggested that uptake was independent of external concentration by measuring the depletion of NO\textsubscript{3}\textsuperscript{-} from the media by Lolium perenne plants at twenty minute time intervals (Clement et al., 1978). As the N concentration fell from 10 \textmu{}M to 1.43 \textmu{}M, the rate of uptake was constant throughout the depletion cycle. The amount of N supplied in order to maintain a constant media N concentration was also plotted over time, indicating constant uptake rates. Not included in this study was the effect of pre-treatment concentration on induction of N uptake. If the plants that were used for this experiment were grown in low N conditions, it is possible that they were already maximizing the use of the high affinity-transport system (HATS) and at lower concentrations could not increase uptake. If the
plants were previously grown in high N concentrations, whereby placement in low N concentrations would induce HATS, constant uptake may not have occurred.

Preferential uptake for NH$_4^+$ over NO$_3^-$ has been shown for Douglas-fir (Kamminga-van Wijk and Prins, 1993), Scots pine (Flaig and Mohr, 1992), maritime pine (Scheromm et al., 1988), jack pine (Lavoie et al., 1992), western hemlock (Knoepp et al., 1993) and white spruce (Kronzucker et al., 1997). These studies produced differing results on the magnitude of NH$_4^+$ preference because of pre-treatment and concentration effects (i.e. the utilization of the low affinity, LATS, and high affinity transport systems). Pre-treatment with either N-form can enhance uptake and N-concentration can determine whether uptake occurs through LATS or HATS. Flux characteristics of NO$_3^-$ and NH$_4^+$ and their compartmentation in white spruce have been studied by Kronzucker et al. (1995 a and b, 1996 and 1997) using radiolabelled N. Their results elucidate the effects of pretreatment and high doses of nitrogen.

As with most higher plants, white spruce has two kinetically distinct transport systems; HATS which operates in a Michaelis-Menten fashion at low external nitrogen concentrations and LATS which operates in a linear unsaturable fashion at high external nitrogen concentrations (Kronzucker et al., 1996). Kronzucker et al. showed that the kinetics of NO$_3^-$ HATS and NH$_4^+$ HATS differed when seedlings were induced with NO$_3^-$ or with NH$_4^+$. Under NO$_3^-$ induction (three days exposure to 100 μM NO$_3^-$), seedlings exhibited two saturable phases, one at external NO$_3^-$ concentrations ([NO$_3^-$]$_o$) ≤ 75 μM and another at [NO$_3^-$]$_o$ between 100-750 μM. When uninduced, spruce exhibited one saturable phase between 200 and 500 μM [NO$_3^-$]$_o$, termed constitutive HATS (CHATS). The first phase in induced seedlings had a similar $K_m$ to the CHATS of uninduced roots but the $V_{max}$ was almost three times higher. This indicated that transport proteins for component I of induced seedlings and CHATS may be similar. The $V_{max}$ and $K_m$ for component II were significantly higher than for the CHATS and reflected a separate inducible transport system (IHATS) (Kronzucker et al., 1995).

When spruce seedlings were given NH$_4^+$ without induction, they showed Michaelis-Menten kinetics similar to when they were given NO$_3^-$, but the $V_{max}$ was substantially higher ($V_{max} = 1.86$ μmol g$^{-1}$ h$^{-1}$ for NH$_4^+$, 0.11 μmol g$^{-1}$ h$^{-1}$ for NO$_3^-$). NH$_4^+$ uptake was also enhanced effectively by NO$_3^-$ (influx increased by 20 to 40% in the low concentration range) but not with NH$_4^+$. Ammonium induction may lead to a negative feedback cycle reducing the ability for increased transport with previous exposure. The negative feedback could also cause the decrease
in NH\textsubscript{4}\textsuperscript{+} influx at higher concentrations that was observed in NO\textsubscript{3}\textsuperscript{-} induced seedlings (Kronzucker et al., 1996).

The LATS is evident in both NO\textsubscript{3}\textsuperscript{-} and NH\textsubscript{4}\textsuperscript{+} uptake, but the external N concentrations at which the saturable phase begins for both ions can vary. When spruce seedlings were grown on NO\textsubscript{3}\textsuperscript{-}, the LATS was visible at 500 μM [NO\textsubscript{3}\textsuperscript{-}]\textsubscript{0} for uninduced seedlings versus 700 μM [NO\textsubscript{3}\textsuperscript{-}]\textsubscript{0} for induced seedlings. This change was accompanied by a slower rate of influx over the range of [NO\textsubscript{3}\textsuperscript{-}]\textsubscript{0} in uninduced seedlings. When spruce seedlings were given NH\textsubscript{4}\textsuperscript{+}, the linear saturation phase began at 400 μM [NH\textsubscript{4}\textsuperscript{+}]\textsubscript{0}, but the slope of the influx of NH\textsubscript{4}\textsuperscript{+} was depressed in the NO\textsubscript{3}\textsuperscript{-} induced seedlings as compared to N-deprived seedlings.

The findings of Kronzucker et al. (1995a, b and 1996) are helpful in interpreting what seem to be conflicting results in the earlier literature. In Douglas-fir, the V<sub>max</sub> for NH\textsubscript{4}\textsuperscript{+} increased in a 100 μM NH\textsubscript{4}NO\textsubscript{3} solution and NO\textsubscript{3}\textsuperscript{-} uptake became negligible (Kamminga-van Wijk and Prins, 1993). NH\textsubscript{4}\textsuperscript{+} uptake may have been enhanced by the NO\textsubscript{3}\textsuperscript{-} present in the NH\textsubscript{4}NO\textsubscript{3} solution which increased the V<sub>max</sub> for NH\textsubscript{4}\textsuperscript{+} but did not change the K<sub>m</sub>. Flaig and Mohr (1992) supplied N to Scots pine at concentrations where LATS would dominate and the 15 mM concentrations of NO\textsubscript{3}\textsuperscript{-}, NH\textsubscript{4}\textsuperscript{+} and NH\textsubscript{4}NO\textsubscript{3} given were well above levels where NO\textsubscript{3}\textsuperscript{-} inhibition would be seen. This illustrates the importance of studying uptake kinetics and the resulting effects on NUE at low external nitrogen concentrations. It is only under these more realistic conditions that differences between the two N-sources are likely to be obvious.

2. N-source effects on uptake, C assimilation, biomass and N assimilation

N source can affect uptake indirectly through the efflux of hydrogen and hydroxyl ions. When NH\textsubscript{4}\textsuperscript{+} and NO\textsubscript{3}\textsuperscript{-} are assimilated, there is a concomitant change in cytosolic pH; NH\textsubscript{4}\textsuperscript{+} assimilation produces at least one H\textsuperscript{+} per NH\textsubscript{4}\textsuperscript{+} ion and NO\textsubscript{3}\textsuperscript{-} assimilation produces almost one OH\textsuperscript{-} per NO\textsubscript{3}\textsuperscript{-} ion (Raven and Smith, 1976). This can be mediated by efflux of excess ions or by utilization of the biochemical pH stat. Maintenance of ionic balance by efflux is manifested by a change in media pH as the plants assimilate each form of nitrogen (Marschner et al., 1991; Kamminga-van Wijk et al., 1993). If the change in the pH of the external solution is extreme, it could affect the uptake of either N ion. The biochemical pH stat maintains cytosolic pH by the synthesis and removal of carboxylic acids, but its effectiveness is limited by the storage capacity of the vacuole. A cytosolic pH in the range of 6-8 activates PEPcase which increases the rate of
CO$_2$ fixation and the synthesis of oxaloacetate. Oxaloacetate is reduced to malate by Malate dehydrogenase and malate is transported to the vacuole where it acts as a counter-ion for cations. Malate can also be incorporated into the cytoplasmic pool of organic acids of the Krebs cycle and traded for another organic acid to be stored in the vacuole. Malic enzyme is activated by a decrease in cytoplasmic pH, often due to an excess of anion uptake (Marschner, 1995). If the biochemical pH stat is utilized to maintain cytosolic pH, this could alter the proportion of CO$_2$ fixation by Rubisco and PEPcase.

The division of CO$_2$ fixation by Rubisco and PEPcase can also be affected by N-source if differing uptake capacities change the availability of N for assimilation. When C isotope discrimination was investigated in N-limited control and NH$_4^+$ pulsed algae, control discrimination was very close to that of Rubisco (30-40 %), while discrimination in NH$_4^+$ pulsed algae was much less (4-11 %), suggesting that the majority of carbon fixation was by PEPcase (Guy et al., 1989). As well, NH$_4^+$ suppressed photosynthetic carbon fixation because N assimilation began to compete with RuBP regeneration for reductant. This caused less available RuBP and thus decreased photosynthesis. Relative to photosynthesis, rates of N-assimilation in these algae are very much higher than in conifers, but the same principles of division between the two carboxylating enzymes should apply. However, the effects on $\delta^{13}$C of gymnosperms should be negligible because the available HCO$_3^-$ in roots is generated by normal dark respiration and is therefore of the same isotopic composition as the plant.

N-source may more directly affect biomass through the bioenergetic cost of the extra assimilatory steps associated with NO$_3^-$ assimilation. Unlike NH$_4^+$, NO$_3^-$ must be reduced prior to assimilation. NO$_3^-$ is reduced by nitrate reductase (NR) followed by nitrite reductase (NiR) and the resulting NH$_4^+$ can then enter the GS/GOGAT cycle. The first step in the cycle is the reduction of glutamate to glutamine by the enzyme glutamine synthase (GS). Glutamine is then either shuttled out of the chloroplast or is used in the nitrification of $\alpha$-ketoglutarate (from the TCA cycle) by glutamate synthase (GOGAT) to replenish glutamate. This starts the GS/GOGAT cycle over again, so that for every N assimilated, one C molecule must be pulled from the TCA cycle. This carbon is replenished by HCO$_3^-$ assimilated by PEPcase (Dennis and Turpin, 1990).

Researchers have proposed that the high energetic cost of NO$_3^-$ reduction may contribute to smaller plant biomass when grown on NO$_3^-$ fertilization (Miflin and Lea, 1976; Raven et al.,
1992). The impact of this cost will depend on the quantity of available light and the site of NO₃⁻ reduction (shoots or roots). In gymnosperms, the majority of NO₃⁻ reduction occurs in the roots, and energy is derived from mitochondrial respiration, the pentose phosphate pathway and malate oxidation in the roots or leaves. When NO₃⁻ is reduced in the leaves, C and N can compete for photochemical energy. Because root reduction is more energetically expensive, the balance between NO₃⁻ reduction in the leaves versus the roots can influence biomass accretion. If NO₃⁻ reduction increases mitochondrial respiration and photosynthesis can not meet the demands of enhanced respiration, then NO₃⁻ fertilization may incur too much of an expense and cause a decrease in biomass. However, in gymnosperms, it is unlikely that low photochemical energy would limit NO₃⁻ assimilation in the roots, but rather low uptake capacity of NO₃⁻ may be directly limiting the formation of photosynthetic proteins (Smirnoff and Stewart, 1985).

N-source may also affect the relative quantity of the N assimilating enzymes. Assimilating enzymes for both NO₃⁻ and NH₄⁺ are regulated by internal concentrations of both ions differentially. NR activity increases with internal NO₃⁻ concentrations (Martin et al., 1981) but is also limited by C availability at higher concentrations (Smirnoff et al., 1985). At low N concentrations, GS and GOGAT respond similarly to the two N-sources (Canovas et al., 1991; Knoepp et al., 1993) or a NO₃⁻ treatment yields the highest GS activity (Vezina et al., 1992; Bedell et al., 1999). Combined, these statements indicate that there may be a positive feedback mechanism between uptake and assimilation for both ions at low concentrations. At higher concentrations, this positive feedback could be interrupted to prevent toxic accumulation of one of the ions.

3. N-source effects on allocation

Allocational differences with N-source may be more accurately related to internal N quantity than the ion. In conifer studies, the difference in allocation appears to be in the relative quantity of the amino acids asparagine (Asn), glutamine (Gln), aspartate (Asp), glutamate (Glu) and arginine (Arg), with varying results on the relationship with N-source. Asp and Asn are substrate and product in the asparagine synthase (AS) cycle which may be one entry point for NH₃ into metabolism. Lavoie et al. (1992) found that NO₃⁻ fed jack pine had higher levels of NH₄⁺, Asp and Gln in the needles than NH₄⁺ fed seedlings. In the stems, the stress related amino acids Arg and γ-aminobutyric acid increased, possibly due to a perceived N depletion when
grown on an exclusively NO$_3^-$ solution. Decreased biomass accumulation in NO$_3^-$ fed plants supports this idea (Lavoie et al., 1992). Flaig and Mohr (1992) fertilized Scots pine with almost four times the amount of both NO$_3^-$ and NH$_4^+$ used by Lavoie et al. and found an increase in the three amino acids which are normally associated with storage proteins (Asp, Gln and Arg). This pool of free amino acids comprised 41% of total nitrogen in seedlings grown in a mix of N sources. The most responsive of the three amino acids was Gln, which is the first product of NH$_4^+$ assimilation and the preferred transport compound in Pinus. Being able to sequester excess nitrogen in these amino acids maintains the N balance of the plant and prevents potential N toxicity. They also found an increase in Asp and Glu in the leaves of NO$_3^-$ fed seedlings. This balance reflects the effect of N-source (or N quantity) on the GS/GOGAT and the asparagine synthase (AS) cycles (Flaig and Mohr, 1992).

N stress can also increase the amount of C allocated to starch or non-photosynthetic tissue when photosynthetic C is not limiting N assimilation. When algae were pulsed with N after a period of N-stress, a greater proportion of carbon skeletons came from starch (Turpin et al., 1991). As well, under N-stress, more carbon is allocated to roots in order to reach new N-sources and increase the surface area for absorption (Birk et al., 1986; George et al., 1999).

In most conifer studies, seedlings show faster growth when grown on NH$_4^+$ versus NO$_3^-$ (Lavoie et al., 1992 and ref. therein and Scheromm and Plassard, 1988 and ref. therein). As is seen above, at equal concentrations of NH$_4^+$ and NO$_3^-$, NO$_3^-$ fed plants perceive stress and allocate nitrogen to proteins involved in growth. When treatments of NH$_4^+$ or NO$_3^-$ are supplied at higher concentrations, more N is allocated to storage forms in NH$_4^+$ fed seedlings whereas in NO$_3^-$ fed seedlings more N is incorporated into immediately used amino acids. This storage capacity found with NH$_4^+$ may contribute to greater conifer biomass if available N is frequently exhausted.

### B. Genetic control of NUE

Genetic control of NUE may reside in N uptake, assimilation or allocation capacities. The difficulty in determining the genetic control of NUE has been in separating the relative importance of the three components across environmental variation. This change in relative importance can frequently cause a g x e interaction. For example, under low N, the inherent ability of the plant to take up N may be more important than the rate of assimilation or how that
N is used and genetic control of uptake is likely to determine NUE. As N supply increases, uptake is not as important because the resource is not limited and maximizing assimilation or utilization takes precedence (Li et al., 1991).

Heritable variation in NUE or associated traits has been found in corn and pumpkin. Eghball et al. (1993) did not find a g x e interaction in mean N-influx (measured as accumulated N over time) between five different corn hybrids in a greenhouse experiment but there was in the field (Eghball and Maranville, 1993). In the greenhouse, three of the hybrids were given four concentrations of NH$_4$NO$_3$: 0, 30, 60 and 90 mg N kg$^{-1}$ soil. Although they showed no significant g x e interaction in mean N-influx, genotypes were significantly different and there was an interaction between root dry weight, average root radius, R:S ratio and root N content measurements. In the field, four hybrids were given a wider range of concentrations of NH$_4$NO$_3$ (0, 60, 120, and 180 mg kg$^{-1}$ soil) but a g x e interaction was only seen in the root length and influx. This study did not show at which concentration the g x e interaction occurred, so it is not possible to ascribe the effect to the idea that at low N concentrations genetic control of uptake will be stronger and at high concentrations the genetic control of assimilation will play a bigger role. The authors also did not give an explanation for the significant effects seen in the field but not in the greenhouse.

Swiader et al. (1991) studied thirteen inbred and hybrid pumpkin genotypes differing on the basis of harvest maturity. When the plants were three weeks old they were transferred to new nutrient solutions which were monitored daily for NO$_3^-$ uptake by measuring loss from the solution. They found that the early maturing genotypes differed more in NO$_3^-$ uptake than the later maturing genotypes, but there was no apparent relationship between parents and progeny. As a group, the later genotypes removed more NO$_3^-$ from the solution and had greater biomass than the early genotypes, but there was no significant difference between early and late genotypes in NUE (C:N ratio) (Swiader et al., 1991). Even though it looked as though there might be a genetic component because of the different responses of the early and late maturing genotypes, the lack of relationship between parents and progeny in NO$_3^-$ uptake was discouraging. Alternatively, the results could indicate the complexity of genetic control of NUE.

Sheppard and Cannell (1995) tested clonal differences in needle nutrient concentration, nutrient allocation to above-ground parts and needle retention in *Picea sitchensis* and *Pinus contorta* trees grown in a lowland fertile site and an upland peaty-gley site. They found that all
three measured variables correlated well with the differences in NUE, and that there were clonal differences in needle nutrient concentration but no significant clone x site interactions. Since N-uptake was not measured, and there was not a g x e interaction, it cannot be assumed that high and low levels of nutrient supply affected NUE differentially. Nonetheless, genetic control of separate facets of NUE (i.e. N allocation and needle retention) indicate the diversity of genetic control of total NUE (Sheppard and Cannell, 1985).

Each of these studies shows that the genetic control of NUE at high and low N fertilization can be divided in multiple ways between variables related to uptake capacity and utilization capacity. Another approach to this may be to study genetic control of NUE when conifer seedlings are fed NH$_4^+$ or NO$_3^-$. Potential genotypic differences in uptake, assimilation and allocation capacities of the two ions could change the NUE ranking within a group of genotypes.

III. Relationship between WUE and NUE

Theoretically, WUE and NUE should trade-off on both an environmental and a genetic basis. Fertilization should increase $A_{\text{max}}$ and WUE but also decrease the C:N ratio. In the absence of allocational differences, genotypes which are adapted to a low nutrient supply may trade-off water use for the efficient use of nitrogen. This should hold true for both short and long-term measures of the two resource-use efficiencies. Unfortunately, the environmental and genetic trade-offs can be confounded by stomatal response to external variables, resource allocation patterns, and genotypic limitations of each resource-use efficiency.

The first published research to substantiate the theory was a comparative study of five California evergreen angiosperms. Field et al. (1983) found a distinct species-level trade-off between the two resource-use efficiencies through the link of $C_i$ levels. There was a positive correlation between $N_{\text{leaf}}$ and $A_{\text{max}}$, but $C_i$ did not vary consistently with this increase. However, $g_s$ did increase with $A_{\text{max}}$, inferring a constant proportional stomatal limitation that was specific to each species. Because $C_i$ levels remained relatively constant for each species in this study, the results imply that there is a positive relationship between $g_s$ and NUE. This idea has not been investigated by many studies because of the strong correlation between $A_{\text{max}}$ and $g_s$. Ecologically, the trade-off implies that plants from drier habitats sacrifice efficient use of N for
more efficient use of water. Plants from wetter habitats maximize their NUE at the cost of transpiration in order to acquire carbon to meet enhanced rates of $A$ (Field et al., 1983).

One means to examine this trade-off, based on known physiological differences in $A_{\text{max}}$, is to compare similar C3 and C4 plants. C4 plants tend to have a higher $A_{\text{max}}$ per unit N than C3 plants because they concentrate CO$_2$ at the site of Rubisco. This concentrating mechanism also allows them to maintain a higher NUE at similar WUE or a higher WUE at similar NUE. Sage and Pearcy (1987), examined two similar C3 and C4 annuals to more precisely understand the physiological differences in nitrogen use. As with the Field et al. (1983) study, $C_i$ did not change perceptively with varying $N_{\text{leaf}}$ for the C3 plant. For both species, there was a positive correlation between $A$ and $N_{\text{leaf}}$, and $g_s$ and $A$, where the C3 plant had consistently lower rates of assimilation and higher conductance than the C4 plant. What is interesting is that both plants had similar $C_i$ at low $N_{\text{leaf}}$, but $C_i$ was inversely related to $N_{\text{leaf}}$ at higher N supply in the C4 plant. This is presumably because of less of an increase in $g_s$ with $A_{\text{max}}$ in the C4 plant. In a dry environment, where maintaining a high WUE is advantageous, keeping $g_s$ low may be more important than letting it vary with $A_{\text{max}}$. These plants typically grow at CO$_2$ saturating levels, which means that their stomata may not be as responsive to N levels within the plant and the strategy for an increased PNUE may be in the investment of proportionally more of the available N into new leaves. A C3 plant may alternatively invest N in proteins to increase the low $A_{\text{max}}$ (Sage et al., 1987).

Patterson et al. (1997) examined this trade-off from an ecophysiological perspective, comparing two spruce species which occur in unique micro-geographic zones at the southern edge of the boreal forest. Black spruce (*Picea mariana*) typically grows in flooded nutrient-poor muskegs, while white spruce (*Picea glauca*) occupies drier upland sites. Rarely do the two species coexist. In a common garden experiment, Patterson grew both species on both high and low water and N applications and measured biomass, photosynthesis, C/N and $\delta^{13}$C. Under N limitation, species did not differ significantly in their NUE. Black spruce, however, had lower NUE at high N and so exhibited greater plasticity in this trait, possibly due to luxury consumption. It also had lower rates of photosynthesis in all but the low N, low water treatment, but was less sensitive to N limitation, which may give it a slight advantage in nutrient limited environments. Under water stress, white spruce exhibited a greater WUE than black spruce only when fertilized. This may be an adaptation to the periodically dry conditions in environments
where white spruce occurs. When both N and water were limited, WUE and NUE for both species were compromised. The most important finding from these experiments was that there was no inherent trade-off in resource use efficiency, but there was a species difference in the plastic trade-off, such that species exhibited greater plasticity in use of the resource that was most limiting in their habitat. From this, Patterson et. al (1997) proposed that evolving a higher NUE or WUE does not necessarily preclude enhancement of the use efficiency of the other resource and that there is an optimization process “whereby each species maximizes the use efficiency of the resource which is most limiting in its normal habitat.”

The lack of an inherent species-level trade-off indicated by Patterson et al. (1997) is likely because of the subtle differences between genotypes or plants in their total physiology. Inherent WUE can differ because of stomatal response to the environment, $A_{\text{max}}$ and SLM. Inherent NUE can differ because of inherent $A_{\text{max}}$, N uptake, assimilation and allocation capabilities, and SLM. When these factors are included, an inherent trade-off between the two resource-use efficiencies becomes harder to find.

One example of this is cited by Meinzer et al. (1992). They compared WUE and NUE in Hawaiian *Metrosideros polymorpha* populations from habitats differing in moisture availability and temperature. Their results indicated that inherent WUE and PNUE did not trade-off because populations maintained constant inherent $A$ while $g_s$ declined over the range of $\delta^{13}$C values. Although they did not mention differences in site fertility, they noted that $N_{\text{leaf}}$ was genetically determined and did not correlate with either $A$ or $g_s$. Thus WUE was determined by water availability in the site at which plants were grown and not N availability. Meinzer et al. (1992) proposed that constant optimal utilization of N maintained PNUE as WUE varied due to $g_s$.

In the summary of the progression of drought stress in a nitrogen deficient plant presented by Reich et al. (1989), the first consequence of a combination of both stresses is stomatal closure which decreases $C_i$. Stomatal closure causes a proportionally greater decrease in $E$ than $A$. A high N plant will exhibit a more drastic change in $A$ because of the higher capacity for biochemical carbon dioxide fixation. Alternatively, as seen in Meinzer et al. (1992) and Livingston et al. (1999), fertilization or an inherently higher $A_{\text{max}}$ could ameliorate the effects of stomatal closure (at the expense of NUE). Under low $N_{\text{leaf}}$, water stress effects on photosynthesis will not be as obvious because carbon fixation is hampered by N stress. Under
severe water stress both \( g_s \) and \( A_{\text{max}} \) decrease because of an increasing influence of biochemical limitations (Reich et al., 1989).

An adaptation to temporal variations in nutrient supply (e.g. spring nutrient flushes) is luxury consumption. Plants may continue a constant absorption of nutrients without a concomitant increase in growth by storing nutrients either as mineral ions or as free amino acids or proteins. This may cause an apparent short-term decrease in PNUE, but over the long term NUE is high. Nutrient storage could also provide a quickly mobilized reserve for periods of nutrient stress (Vitousek, 1982; Elliott and White, 1994). Birk and Matson (1986) in studying loblolly pine (Pinus taeda L.) showed that carbon can also be stored during nutrient deficiency as starch when maintenance demands are less than carbon production. When resources are plentiful, the stored carbon and nitrogen can be readily mobilized and used for growth (Chapin, 1980; Birk and Matson, 1986).

Modifications in allocational patterns induced by water or nutrient stress can also influence the trade-off on a long-term basis. Under these stresses, plants increase their R:S ratio as well as modify the shape and size of the leaves. Plants with greater root mass or high SLM may have less leaf area for transpiration and photosynthesis. If soil N content is low, producing thicker leaves may not only help in avoiding potential water loss, but may also make the plant less susceptible to herbivory and trampling. PNUE measured on an area basis then may be quite low, but long term NUE and WUE would be high if other dangers are avoided (Fichtner and Schulze, 1992). DeLucia and Schlesinger (1995) compared evergreen and deciduous swamp shrubs and found that the high SLM of evergreens correlated negatively with PNUE but positively with \( \delta^{13}C \) and therefore WUE. They attributed the correlations to devotion of the N to non-photosynthetic functions such as defense, and to a high cell wall resistance to diffusion (DeLucia et al., 1989).

IV. Stable Isotopes in Physiological Research

The use of stable isotopes at natural abundance levels has provided a tool to investigate plant physiology. The process of discrimination either through physical (e.g. diffusion or isotope equilibrium effects) or biochemical (e.g. enzymatic reactions) processes changes the isotopic composition of the plant component relative to the source. This change in isotopic composition can indicate the dominant reactions involved in metabolism of that element. Natural abundance
levels of the stable isotopes of carbon, oxygen, hydrogen and nitrogen have all been used in physiological research. Although the results are often very precise, the utility of the information is enhanced by coupling the data with other techniques. Thus, not only should one element be measured, but enzyme activity or quantity, elemental concentration or a second stable isotope should also be measured. By using multiple techniques the verity of the theories resulting from stable isotope composition can be supported.

A. Using natural abundance levels of nitrogen stable isotopes in physiology

Application of stable nitrogen isotopes to physiological research is relatively novel, with the exception of studies on diazotrophy. In fact, few connections between $\delta^{15}$N and other physiological traits have been recognized. Discrimination factors associated with particular enzymes involved in N assimilation have been determined and $\delta^{15}$N values have been used to trace the N source from which the plants are feeding, but a conclusive theory on fractionation and how it relates to stress physiology has been hinted at only within the last few years.

Notation to describe N isotope discrimination is similar to carbon, but the standard is air:

$$\delta^{15}N \%o = \frac{^{15}N/^{14}N_{sample} - ^{15}N/^{14}N_{standard}}{^{15}N/^{14}N_{standard}} \times 1000$$

(5)

The atom percent abundance of $^{15}$N in air is approximately 0.3663 %%. Therefore, a 1%o change in the $\delta^{15}$N of a plant represents less than a 0.0004 change in the per cent abundance of $^{15}$N. The Rayleigh distillation model has been used in various forms to calculate discrimination or enrichment factors dependent on whether the substrate or product is analyzed. For example, the model can describe the change in $\delta^{15}$N values of the substrate as it is assimilated, assuming constant fractionation at all external substrate concentrations:

$$\varepsilon = \ln \left( \frac{R}{R_0} \right) \times 10^{-3}$$

(6)

where $\varepsilon$ is the enrichment factor of the product relative to the substrate, $R/R_0$ is the ratio of the isotope abundances ($^{15}N/^{14}N$) of the substrate at any time relative to time 0 and $f$ is the fraction of unreacted substrate at any time during uptake (Mariotti et al., 1981). The notation is similar to $\Delta$, but $\Delta$ is given as the isotopic composition of the substrate relative to the product, where $\varepsilon = R_p/R_s - 1$ and $\Delta = R_s/R_p - 1$. This generally causes $\varepsilon$ to be negative and $\Delta$ to be positive. $\varepsilon$ and $\Delta$ can be equated mathematically by using the discrimination factor, $D$ (Guy et al., 1993).
\[ \varepsilon = -D \quad \text{and} \quad \Delta = \frac{D}{1-(D/1000)} \]  

Similar to C isotope discrimination in photosynthesis, N fractionation could occur at two steps, uptake and assimilation. At each step, fractionation may also be concentration dependent. For instance, if the two mechanisms of uptake, HATS and LATS, were to have different discrimination values, the point at which uptake changes from HATS to LATS would determine plant discrimination. Hoch et al. (1992) first presented this hypothesis to account for discrimination by a marine bacterium. They fed the bacterium \(< 0.1 \text{ mM } \text{NH}_4^+ \) and \(>1 \text{ mM } \text{NH}_4^+ \) and discovered a shift in isotopic composition that correlated with the shift from active transport of \(\text{NH}_4^+ \) to diffusion of \(\text{NH}_3 \). Despite this correlation they could not conclude that fractionation was causally linked to uptake. In the concentration range where diffusion was the mode of uptake, isotopic equilibrium was reached intra- and extra-cellularly and the assimilating enzyme became the rate-limiting reaction responsible for fractionation. Under the concentration range of active transport, fractionation was greater than would be expected for the assimilating enzyme and it was proposed that uptake contributed to enhanced discrimination (Hoch et al., 1992).

Yoneyama et al. (1991) also came to the conclusion that N uptake contributed to whole plant discrimination when fertilized with \(\text{NH}_4^+ \). They attributed fractionation in uptake to both the isotope equilibrium effect (which causes a 20 % enrichment of \(\text{NH}_4^+ \) nitrogen) and the difference between active transport of \(\text{NH}_4^+ \) and diffusion of \(\text{NH}_3 \). Using wild-type cyanobacteria (Synechococcus PCC 7942) fed with \(\text{NO}_3^- \), and a mutant deficient in assimilating both \(\text{NO}_3^- \) and \(\text{NO}_2^- \) fed with \(\text{NH}_4^+ \), they found that discrimination was much higher in the \(\text{NH}_4^+ \) fed bacteria. In addition, they compared the discrimination values to rice plants which were fed with 3.57 mM and 0.71 mM \(\text{NH}_4^+ \) at a pH between 4 to 6.5. Both the wild-type and mutant cyanobacteria fed with 8.75 mM \(\text{NH}_4^+ \) and grown at pH 8 showed greater discrimination than the rice plants. The authors proposed that at pH 8, 5% of the N is \(\text{NH}_3 \) and the large depletion of \(^{15}\text{N} \) was a result of isotopic equilibrium and kinetic isotopic fractionation. At the lower pH and lower concentration, discrimination was primarily determined by active transport of the protonated form of the ion.

In a second publication by the same authors, on \(\text{NO}_3^- \) fertilization, there was no apparent discrimination in uptake or assimilation at 1.2, 3.0 or 12 mM \(\text{NO}_3^- \) (Yoneyama and Kaneko,
1989). Regardless of the rate or the concentration supplied, whole plant $\delta^{15}N$ was not significantly different from the salt $\delta^{15}N$. However, when N concentration of the medium was plotted over time, the lower concentrations reached almost 0 mM after one week and the upper concentration decreased only slightly from 12 mM to 10 mM. It is possible that at the lower concentrations, discrimination in net uptake was not evident because all of the NO$_3^-$ was taken up and at the higher concentrations, slight discrimination was masked by a build-up of $^{15}N$.

Studies in fractionation associated with the assimilating enzymes in vitro are more conclusive. In 1985, Ledgard et al. determined discrimination associated with NO$_3^-$ reduction. They described four places at which fractionation could occur: (1) reduction of NO$_3^-$ to NO$_2^-$ by NR; (2) diffusion of NO$_2^-$ into the chloroplasts; (3) reduction of NO$_2^-$ to NH$_3$ by NiR; and (4) diffusion of NH$_3$ out of the chloroplasts.” By comparing the $\delta^{15}N$ values of NH$_3$ produced by isolated chloroplasts and a reconstituted system containing cytosolic extracts and chloroplasts, they were able to discern where fractionation occurred. The first step (reduction of NO$_3^-$) occurs in the cytosol and its activity is one fifth to one twentieth that of NiR. It was the only step of the four that was not included in the isolated chloroplasts. The isotope signature of the NH$_3$ produced in the reconstituted system was not significantly different from the NH$_3$ produced by the isolated chloroplasts. This indicated that discrimination in NO$_3^-$ assimilation occurs only at the reduction of NO$_3^-$ by NR. Beyond this point, all substrate was used in the subsequent reactions and therefore no fractionation occurred. The $\Delta \delta^{15}N$ value ($\delta^{15}N_{\text{product}} - \delta^{15}N_{\text{substrate}}$) of NR in spinach leaves was -15 %° (Ledgard et al., 1985).

Glutamine synthetase is the primary NH$_4^+$ assimilating enzyme and would likely determine fractionation in NH$_4^+$ assimilation. Yoneyama et al. (1993) isolated chloroplastic glutamine synthetase (GS2) from spinach leaves and incubated the enzyme with 20 mM or 10 mM NH$_3$ and glutamate at differing combinations of time and temperature. They measured the final quantity and stable isotope composition of unreacted NH$_3$, amide (glutamate) and glutamine and found that the glutamine produced was lighter than the NH$_3$ supplied. $\Delta^{15}N$ for GS2 was $-16.5 \pm 1.5$ %°. Because the substrate supplied was NH$_3$, this figure did not incorporate the equilibrium isotope effect (Yoneyama et al., 1993).

From these two studies on NR and GS2 and references therein, it would seem that fractionation by the initial assimilating enzymes is similar for both NO$_3^-$ and NH$_4^+$. In both cases, activity of the enzyme represents the rate-limiting step for N assimilation (Robinson et al.,
The amount of fractionation in vivo seems to depend on substrate concentration. In the publication by Yoneyama et al. (1991), rice plants were fed high and low NH$_4^+$ concentrations for 20 or 35 days. At 20 days, there were no significant treatment differences in $\delta^{15}$N, but all plants were lighter than the source. At 35 days, high NH$_4^+$ fed plants had become 3-5 %o lighter, while the low NH$_4^+$ fed plants had become 3-5 %o heavier. This possibly reflects differences in enzymatic discrimination due to external N concentrations.

A second level of discrimination occurs within plants between plant parts or processes. For example, ability to assimilate NO$_3^-$ in both the roots and the shoots could cause transport of relatively heavy residual N to the shoot. When tomato plants were fed very low (50 µM) concentrations of NH$_4^+$ and NO$_3^-$ there was no difference in whole plant $\delta^{15}$N composition from either source because all substrate was consumed (Evans et al., 1996). However, intraplant differences were significant in the NO$_3^-$ fed plants. Leaves were heavier and roots were lighter than whole plant $\delta^{15}$N, but both leaf and root $\delta^{15}$N got closer to the whole plant value as the plant aged. The authors proposed that the convergence was due to increased partitioning of NO$_3^-$ assimilation to the shoot as the plants aged. Similar results were found when rice plants were fed with the NO$_3^-$ concentrations mentioned above (Yoneyama et al., 1989), in that roots were lighter than leaves, petioles and midribs.

There has been an emphasis in oceanography on understanding N isotope fractionation during uptake by marine bacteria and phytoplankton. Within this discipline, researchers have intended to use the natural abundance ratios in underlying sediment to trace past oceanic productivity. To this end, Waser et al. (1998, 1999) have experimented with differing N-sources and with N-deprivation to alter phytoplankton $\delta^{15}$N. In 1998, they presented their first results on N-deprivation followed by N addition. As expected, discrimination decreased as substrate N concentration decreased, until $\delta^{15}$N$_{PON}$ (PON- particulate organic nitrogen) equaled that of the combined substrates (NO$_3^-$, NH$_4^+$ and urea). When N was added after N-starvation, there was very little fractionation and $\delta^{15}$N$_{PON}$ again equaled that of the combined substrates. Waser et al. (1998) suggested that PON was enriched in $^{15}$N because isotopically light amino acids had been effluxed. In 1999, they published again on N-starvation in phytoplankton and produced similar results. In this experiment, phytoplankton were fed 30 µM NH$_4^+$ in three sequential pulses. During the uptake of N in the first pulse, $\delta^{15}$N$_{PON}$ became lighter during the first 128 hours and then became more enriched as the NH$_4^+$ concentration dropped to 0 µM. During the second N-
pulse, all of the nitrogen was depleted within eight hours and fractionation initially increased, then decreased with external concentration. A six hour time period elapsed between total exhaustion of NH$_4^+$ in the second pulse and addition of NH$_4^+$ in the third pulse. Uptake rate was fastest during the third pulse, and discrimination was non-existent. From these results, Waser et al. re-stated that N status of the phytoplankton can affect discrimination and efflux, but revised the theory of exotic light N efflux. In this publication, they suggested that under N-starvation there is less efflux and more complete utilization of the inorganic N taken up by the phytoplankton (Waser et al., 1999).

Environmental stress experiments have also been done using genotypes of barley. When plants were salt stressed (Handley et al., 1997) or water stressed (Robinson et al., 2000) their $\delta^{15}N$ was lighter than the control. N-stress appeared to have the same effect, but the experimental designed was flawed in this respect. In the first publication, using salt stress, treatment related differences in $\delta^{15}N$ were attributed to down-regulation of the assimilating enzymes due to stress. In the second publication, using N and water stress, the authors proposed that the lighter $\delta^{15}N$ values were due to the loss of amino acids. They hypothesized that when plants are stressed, loss of N from the roots is restricted to one or two amino acids which have "exotic" $\delta^{15}N$ values. That is to say, they are exceptionally light in isotopic composition. Unfortunately, because such small changes in media N concentrations would be difficult to see using standard concentration measurement techniques, they presented little evidence for organic N efflux.

More notably, in these two publications, the authors have demonstrated genotypic control of $\delta^{15}N$. They are the first research group to publish such results and it introduces new possibilities for screening genotypes on the basis of their $\delta^{15}N$ value. Before screening can be useful, the physiological basis for fractionation must be clarified. The literature indicates that 1) there is no apparent discrimination in NO$_3^-$ uptake, 2) fractionation associated with NH$_4^+$ uptake may be a result of the isotope equilibrium effect, 3) discrimination by each of the primary N assimilating enzymes is similar, 4) fractionation can depend on external N concentration and 5) isotopic composition of plant parts can differ depending on the location of the assimilating enzyme. By integrating these ideas researchers can create a theory on $^{15}N/^{14}N$ fractionation in plants that might be useful for genotype screening for NUE.
MATERIALS AND METHODS

I. Plant Material and Germination

Plant material was selected based on the ten full-sibling families used by Sun et al. (1996) and Livingston et al. (1999). Seeds were requested from the Ministry of Forests (Victoria, BC), but only nine of the original ten controlled crosses could be obtained. A tenth full-sib family, with similar lineage, served as a replacement.

Table 1. List of full-sib family crosses and their origin. Letters represent area of origin, where PG = Prince George selection unit, EK = East Kootenays selection unit and ENA = Eastern North America selection unit.

<table>
<thead>
<tr>
<th>#</th>
<th>Female x</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>PG 1</td>
<td>PG 79</td>
</tr>
<tr>
<td>1</td>
<td>PG 161</td>
<td>PG 21</td>
</tr>
<tr>
<td>2</td>
<td>PG 3</td>
<td>EK 13</td>
</tr>
<tr>
<td>3</td>
<td>PG 129</td>
<td>ENA 866</td>
</tr>
<tr>
<td>5</td>
<td>PG 145</td>
<td>PG 5</td>
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<td>6</td>
<td>PG 126</td>
<td>EK 57</td>
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<tr>
<td>7</td>
<td>PG 21</td>
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<td>8</td>
<td>PG 171</td>
<td>PG 41</td>
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<tr>
<td>9</td>
<td>EK 30</td>
<td>ENA 872</td>
</tr>
<tr>
<td>10</td>
<td>EK 13</td>
<td>EK 29</td>
</tr>
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*Numbers listed correspond with the identification numbers used in Sun et al. (1996). Note the number 0 replaces number 4. These numbers are used throughout the thesis when identifying families.

Six of the eight females from the Prince George region were grafted scions from plus trees. The accessions of four of these plus trees are actively used as breeding trees because of outstanding physiological traits. The remaining families were deliberately chosen to have poor productivity in field tests. For further details refer to Sun et al. (1996).

For all experiments, seeds were imbibed under running cool tap water for twenty-four hours and then stratified by storing in wet paper towel in plastic bags at 4°C for three weeks. After stratification, seeds were sown in 10 cm³ Cone-tainers (Stuewe and Sons, Corvallis, Oregon) in sterile pre-made potting media (1 part peat: 0.5 parts perlite and 600 g dolomite) and covered with a thin layer of forestry gravel. Each of the Cone-tainers was filled with a similar amount of potting media to prevent soil density effects on germination, and labeled by genetic cross on the outside. Seventy-five Cone-tainers, each sown with two seeds, were used for each of the ten families. After sowing, each of the Cone-tainer racks was watered and covered tightly.
with cellophane to reduce evaporation and placed in a growth chamber (Conviron CMP 3023, 3244, Winnipeg, Manitoba) at 16h/8h light/dark, 20° C/14° C day/night and ~ 35% relative humidity. Light levels in the growth chamber were approximately 360 –400 μmol m⁻² s⁻¹ PPFD. The seeds were sprayed twice daily with distilled water until germination.

After germination, seedlings were watered with a fertilizer solution containing 100 μM N + 1/10 modified Johnson’s solution (Appendix 1) for the first 6 weeks and 1.5 mM N + 1/10 modified Johnson’s for the last two weeks before being transplanted into hydroponics. Originally, seedlings were given only 100 μM N solution + 1/10 modified Johnson’s solution to maintain hydroponics treatment conditions in soil, but this concentration was too low for soil (as evidenced by slow growth and chlorosis) and was increased in the last two weeks. Nutrient concentrations were 1.4 ppm N, 6.2 ppm P, 23.5 ppm K, 2.4 ppm Mg, 16.04 ppm Ca, 22.5 ppm S and 0.19 ppm micro-nutrients (Cl, B, Mn, Zn, Cu, Mo, and Fe). Seedlings were fertilized every second day to the drip point.

II. Treatments in Hydroponics

A. N-source experiment (Exps. 1 and 2)

The N-source experiment was performed twice, once in Fall 1998 (Exp.1) and a second time in Winter 1999 (Exp.2). The materials and methods were similar for both trials, but there were some unavoidable differences that affected the health of the seedlings. Outside of seasonal effects (e.g. changes in atmospheric CO₂ concentration), the two different growth chambers used noticeably affected the temperature of the nutrient solution and possibly other factors as well.

After 8 weeks, eight seedlings from each family were transplanted into the treatment-respective hydroponics boxes. The boxes were plastic tubs (16 L, 46 cm x 33 cm x 11.5 cm) with black Plexiglass lids from which the seedlings were suspended using styrofoam bungs. Each of the seedlings was first washed in cool, running tap water to remove all soil and then suspended in a treatment box containing 14 L of 100 μM N + 1/10 modified Johnson’s solution for 60 days. The nitrogen treatments were ammonium sulfate, (NH₄)₂SO₄, calcium nitrate, Ca(NO₃)₂, and ammonium nitrate, NH₄NO₃, with two boxes for each treatment. The boxes were aerated using ambient air from within the chamber to both increase circulation and maintain dissolved oxygen levels above 90% air saturation. Powdered CaCO₃ was added to each box as necessary to maintain pH near neutral (pH = 5-7.8). Each of the boxes was placed in the growth
chamber with identical day/night conditions as for germination. The temperature of the solution in the boxes was maintained by the ventilation system in the floor of the growth chamber and by cooling coils underneath the boxes. Reflective tape was used to increase the albedo of the box lids. The solution temperature was 20-22°C for Experiment 1 and 24-25°C for Experiment 2.

Nutrient concentrations were maintained by pumping in concentrated modified Johnson's solution and by daily manual additions. The intent was to compensate for nutrient uptake with the peristaltic pumps, but this proved more difficult than expected. As a result, on most days the nitrogen concentration was assessed it had decreased below target concentrations despite continuous additions through the pump system. Concentrations were amended by pipette and pump rates were again adjusted to try to compensate for uptake. The six peristaltic pumps (FPU100 Series Omegaflex, Omega Engineering, Inc.) were interfaced to a 486 personal computer and controlled by Labtech software.

Five times a week, medium NH$_4^+$ and NO$_3^-$ concentrations were assayed using the phenolhypochlorite method (Solorzano, 1969) and perchloric acid method (Cawse, 1967), respectively. Color yield of the NH$_4^+$ assay varied by almost ten percent, whereas the NO$_3^-$ assay proved more stable and reproducible. Ammonium concentrations also deviated more from the desired concentration because the NH$_4^+$ uptake rate was higher and increased more rapidly than NO$_3^-$ uptake. After determining the N concentration of each of the treatment boxes, the molarity was adjusted as above. This method changed the ratio of macro- and micro-nutrients to N, but in all cases N remained limiting.

The boxes were cleaned once a week to prevent microbial growth and nutrient solutions were replaced to raise all nutrient levels to their original concentrations. Again, powdered CaCO$_3$ was added to neutralize the solutions. Boxes were also rotated randomly within the growth chamber when the nutrient solution was refreshed to reduce positional effects within the chamber. For the first three weeks in hydroponics, dead seedlings were replaced with seedlings that were still growing and regularly fertilized in the cone-tainers. After three weeks, dead seedlings were removed from the treatment box but not replaced because at this point size discrepancies could have affected treatment results.

Fungal infection (Exp.2) was treated with the fungicide Benomyl. Aquatic fungus was first visible three weeks into the experiment and covered the roots of all treatment boxes approximately equally. Six grams of Benomyl was added daily for one week. At the end of one
week the dead fungus and fungicide were manually washed off the root surface. Large pieces of root material were lost but contamination did not re-occur.

**B. N-supply experiment**

Treatment conditions and routine maintenance in the N-supply experiment were similar to the N-source experiment except for the hydroponics treatments imposed. In this experiment, the two treatments were a steady-state supply of 200 μM NH₄⁺ + 1/10 modified Johnson’s solution and a draw-down treatment where NH₄⁺ was not re-supplied until there was a negligible amount left in the box. Pre-treatment conditions in Cone-tainers were identical to the N-source experiment, but all seedlings were fertilized with NH₄⁺. Growth chamber conditions, hydroponics aeration, pH maintenance and temperature (24-25°C) were as already described.

Peristaltic pumps delivered NH₄⁺ to the two steady-state boxes. The target concentration was also maintained by pipetting NH₄⁺ into the boxes on a daily basis. In the draw-down treatment, NH₄⁺ was either pipetted into the box when NH₄⁺ concentration reached zero, or if it was within 24 hours of a full nutrient solution replenishment then the NH₄⁺ concentration was amended when the box was replenished.

**III. Measurements**

**A. Chlorophyll fluorescence**

At the end of the 60-day treatment period, several measurements were taken before harvesting the plant tissue. Chlorophyll fluorescence was measured on five seedlings in each box in Exps. 1 and 2 with an OS-500 Modulated Fluorometer (PP systems, Haverhill, Massachusetts). A leaf clip was placed on a random branch of the seedling to block out light from the growth chamber. After five minutes of dark adaptation time, the probe was placed in the leaf clip and minimal fluorescence (Fo) was recorded for 20 sample points. Next, saturating light (peak wavelength = 670 nm) was emitted to test the maximum fluorescence (Fm) of the tissue. The ratio of the variable fluorescence, Fv, (where Fv = Fm - Fo) to the maximum fluorescence (Fv/Fm) was recorded as an indicator of potential photoinhibition between treatments.
B. Gas exchange

Periodically during the last week of treatment, random seedlings from each treatment box were selected for gas exchange measurements. For Exp. 1, seedlings were chosen from all families. Because this did not provide many replicates of both treatment and full-sib family, seedlings were selected from only three families in all treatments for Exp. 2 and the N-source experiment. Measurements were made at saturating PAR (750 $\mu$mol m$^{-2}$ s$^{-1}$), ambient CO$_2$ concentration (360 ppm), 45% RH and a leaf chamber temperature of 23-25$^\circ$C using an ADC LCA-4 open system (Hoddeson, England). Flow rate through the chamber was altered to maintain $\Delta$CO$_2$ $<$ 40 ppm and $\Delta$RH $\approx$ 15%. Measurements were made in the evening before the night cycle had begun.

After taking gas exchange measurements on 83 seedlings in the N-source experiments and 48 seedlings in the N-supply experiment, values were screened for factors that could confound plant performance in the leaf cuvette. These factors were $C_a$ $<$ 300 ppm, $\Delta$RH $<$ 5%, PAR $<$ 700 $\mu$mol m$^{-2}$ s$^{-1}$, leaf area $>$ 16 cm$^2$, and $g_s$ $=$ 0. The above values represent conditions that are beyond the normal environmental range or impair the accuracy of the equipment. In the case of leaf area, values above 16 cm$^2$ resulted in too much self-shading. Screening for these factors reduced the database substantially to 42 data points for the N-source experiments and 34 for the N-supply experiment.

C. Plant harvesting

At the end of the 60-day period, plants were harvested from each of the treatment boxes. Roots were rinsed in distilled water and the stem was severed just below the oldest needle with a razor blade. The root and shoot were packaged separately in foil packets, frozen in liquid nitrogen and stored at -80$^\circ$C. Before grinding, seedlings were freeze-dried (Labconco Lyphlock 6 liter freeze dry system, Kansas City, MO) in the foil packets to remove all water.

D. Biomass measurements

Three measures of growth were taken: seedling height, ratio of root dry weight to shoot dry weight (R:S ratio) and total dry weight. Seedling heights were measured from the first needle at the base of the seedling to the tip of the topmost needle. R:S ratio and dry weight were
measured after the seedlings had been freeze-dried. All seedlings were kept in a dessicator before measurement.

**E. C:N and stable isotope analyses**

After weighing, roots and shoots were combined and reduced to sub-micron particle size by grinding with a Wiley Mill to pass through a 40 mesh screen, and then pulverizing in a planetary ball mill (Pulverisette, Fritsch GMBH, Germany). Equal weight samples of the eight replicates from each cross within each box were pooled into one composite sample for analysis. Samples were combusted in a Europa ANCA-GSL preparation module. The liberated gas then passed through a Europa Hydra 20/20 ratio mass spectrometer (UC Davis Stable Isotope Facility) for analysis of both carbon and nitrogen stable isotopes. Duplicate samples of random seedlings were sent to UC Davis for reference analysis. In addition, samples of the reference material used by UC Davis were also analyzed at the UBC Dept. of Earth and Ocean Sciences. Isotopic composition of the salts used in the medium were analyzed at UC Davis. The salt $\delta^{15}N$ values used were the mean of four samples and were as follows: $(\text{NH}_4)_2\text{SO}_4 = 0.365 \% \sigma$, $\text{NH}_4\text{NO}_3 = 2.3925 \% \sigma$ and Ca(NO$_3$)$_2 = 0.085 \% \sigma$. All data were corrected for the $\delta^{15}N$ composition of the salt by subtracting $\delta_s$ from $\delta_p$ (where s and p refer to source and plant) and as such plant $\delta^{15}N$ is represented by $\Delta\delta^{15}N$.

**IV. $^{15}N$ Depletion Analysis for N-Supply Experiment**

Stable isotope composition of the nutrient solution in the N-supply experiment was analyzed periodically during two draw-down cycles and on two subsequent mornings in the steady-state treatment boxes. The draw-down treatment boxes were sampled four times in a 10 h period at estimated NH$_4^+$ concentrations of 200, 100, 50 and 10 $\mu$M. After samples were collected, the $^{15}N$-$\text{NH}_4^+$ quantity in the water in the treatment boxes was determined by the $^{15}N$ membrane diffusion method (Holmes et al., 1998). The method, as outlined by Holmes et al. (1998), relies on the volatilization of NH$_3$ out of the solution and subsequent deposition on suspended acidic glass fiber filters. The glass fiber filters are analyzed for $\delta^{15}N$.

Collected samples of hydroponics medium were preserved by adding H$_2$SO$_4$ to acidify the solution to pH = 2.0. Since the pK for ammonium disassociation is 9.25, the protonated form should be stable and not volatilize as NH$_3$ (Shearer et al., 1989). At this point, samples were of
varying volumes dependent on the concentration. The membrane diffusion process requires that all samples are a consistent volume (40 ml) and contain similar quantities of nitrogen (112 µg). In order to achieve this requirement larger samples of a lower concentration were concentrated under acidic conditions using a flash evaporator (Buchi Rotovapor Re III, Switzerland). This method proved to be the fastest and most reliable method for retaining the NH$_4^+$ in the sample and preventing contamination. To ensure that nitrogen was not gained in the process a blank solution was also reduced. The blank was created by bubbling basic distilled water (pH= 10.711) with helium for five minutes to volatilize and release all NH$_4^+$ followed by reacidification. In addition, a 40 ml blank was also used in the diffusion process.

Once the samples had been concentrated, the solution was made basic again by the addition of MgO. Acidified glass fiber filter (GF/F) discs enclosed in Teflon membrane traps were placed in plastic, re-sealable sample bottles to trap ammonia. After six days of incubation on a rotary shaker at 40° C, the discs were removed and dried in a desiccator with a 20 ml beaker of concentrated H$_2$SO$_4$ for 48 h. Once dry, the discs were wrapped in tin squares and combusted in a Carlo Erba model 1106 CHN analyzer. The resultant N$_2$ gas passed into a Fisons Na 1500 ratio mass spectrometer (UBC, Department of Earth and Ocean Sciences) for $\delta^{15}$N analysis.

V. Statistical Analysis

Data were analyzed through a series of one-way analyses of variance (ANOVA) (SAS Inc. Cary, NC) where each variable (biomass, R:S, C:N, $\delta^{13}$C and $\delta^{15}$N) was tested in a split-plot design. The main effects or factors were treatment, replicate and their interaction (Error I) and the subplot contained family, family x treatment interaction and the replicate effect on the family x treatment interaction (Error II). A completely randomized design was justified over a randomized complete block design because of biological interference (fungal infection in Exp. 2) and differences in experimental methods. Based on components of variance, all factors could not be tested against the total error term and were tested against the error terms indicated in Table 3. Among the factors, there was not a suitable error term for treatments and pseudo-F tests were created using the mean square of Error I, Error II and the treatment x family error. Degrees of freedom for treatment were calculated similarly (Appendix 2). The use of a pseudo-F prevented comparison of treatment means through normal multiple comparison tests. To replace this, data were plotted in a histogram with standard error bars to indicate differences between
means. Differences between family means were tested using a Tukey multiple comparison procedure.

Prior to the analysis of variance, each variable was tested for normality and homogeneity of variances both through examination of histograms and stem and leaf plots and through various statistical tests of skewness and kurtosis. Those variables that did not meet these criteria were log transformed and statistical analysis was performed on the new values, but original values were used to produce figures. Pearson correlation coefficients between variables were estimated using transformed variables as appropriate. For the environmental correlations all data were combined within each experiment. For the family correlations, treatments were separated and families were blocked within treatment and replicate.

The gas exchange data were treated somewhat differently. Data were blocked as N-source and N-supply experiments, the N-source block containing all data from Exps. 1 and 2 in order to increase the sample size. The majority of this data was from Exp. 2 because of errors made during measurements taken on seedlings from Exp. 1. The assimilation rate observations from the N-supply experiment, the \( E \) observations from both experiments and the \( g_s \) observations from both experiments were not normally distributed and were subsequently log transformed to produce a normal distribution. Because there were only three factors (treatment, family and treatment x family), no pseudo-F tests were created and all factors were tested against the error term. Where there was a significant difference in treatment or family means, a Scheffe multiple comparison test was used.
RESULTS

The intent in running all three experiments in a growth chamber using hydroponics was to try to control as many factors as possible. By controlling environmental factors, one can discern more clearly specific treatment effects without confounding interactions. Unfortunately, even in controlled experiments, there can be unforeseen environmental differences that can affect treatments. For instance, in the N-source experiment, replicates of the same treatment within Experiments 1 and 2 were significantly different in almost every measured phenotypic variable. Between those two experiments, most of the measured phenotypic variables were significantly different due to the fungal contamination. These uncontrollable environmental factors must be considered when analyzing the results both from the standpoint of the resiliency of the genotypic determination of particular traits and for the purpose of understanding the strength of a nitrogen treatment effect over other variables.

The intended and unintended differences between experiments merit presentation of each of the results individually, despite having measured the same phenotypic traits. Treatment and family effects on traits are addressed first, followed by trait correlations. Although individual trait responses to treatment are interesting, invariably that treatment must have an interrelated effect on other traits as well.

I. N-Source Experiment

A. Chlorophyll fluorescence

If a seedling is photoinhibited, maximal fluorescence (Fm) decreases because more energy is lost as heat to prevent further damage to the photosynthetic apparatus. This provides a quick method for analyzing potential damage to photosynthesis from nutrient stress. A healthy plant typically has a Fv/Fm ratio of 0.83. In Experiment 1 mean Fv/Fm was 0.777 and all treatments were similar. In Experiment 2, mean Fv/Fm was 0.601 and the lowest value was 0.246 in one of the NH₄NO₃ replicates.

B. Total biomass and allocation

Biomass differences among treatments in the N-source experiments were significant (α(2)= 0.05) (Appendix 2, Table 1). Total biomass data were log transformed to produce a
normal distribution of the variances and pseudo F-tests were created to remove both replicate and family variation. The highly significant Error I term in Exp. 1 indicates that replicates of the same treatment were more different than between treatments. Although the correlation coefficient between replicates of the same treatment in Exp. 1 was significant for all treatments ($\alpha = 0.05$), the difference in replicate means in both the NH$_4$NO$_3$ and NH$_4^+$ treatments may have caused the treatment x replicate effect (Error I). Nonetheless, when represented graphically (Fig. 1a) the mean seedling biomass in the NH$_4$NO$_3$ and NH$_4^+$ treatments was greater than the NO$_3^-$ treatment. In Exp. 2, Error I was not significant and a higher biomass in the NH$_4$NO$_3$ and NH$_4^+$ treatments was not as pronounced (Fig. 1b). As a whole, the seedlings in the trial that experienced a fungal infection were smaller than the healthy seedlings in Exp. 1 (pers. obs.). Based on this, the results presented will focus on Exp. 1, with occasional mention of Exp. 2 data.

Family differences in total biomass were significant and larger families maintained their rank in all three treatments. There was some variation in the middle of the range of biomasses for each treatment, but the families which represented the highest (1, 3, 10) and lowest biomasses (0, 2, 6) were consistent for all treatments and both experiments. Significant Pearson correlation coefficients for biomass between each of the treatments in Exp. 1 and in two of the three treatment comparisons in Exp. 2, indicate strong genotypic control of this trait (Appendix 5, Table 1). In Exp. 1, it is clear that there is some clustering in the middle, but that the families on either end are significantly different (Fig. 2a and Appendix 3). Such clear grouping is not evident in data from Exp. 2 because there was a family x treatment x replicate interaction (Error II), preventing any distinction other than a significant difference between the means of families 10 and 5. In both experiments the range of biomasses covered by the families was wider for NH$_4^+$ and NH$_4$NO$_3$ than for NO$_3^-$ alone.

Nitrogen treatments did not have a significant effect on R:S ratio in the N-source experiments (Appendix 2, Table 2). From Fig. 1a it appears that seedlings in the NO$_3^-$ treatment had the highest R:S ratio, whereas the NH$_4^+$ and NH$_4$NO$_3$ treatment R:S ratios were similar to one another, but the analysis of variance and T-tests did not support these differences. The R:S ratio of the families clustered in a low (1, 3, 5, 6, 7 and 10) and a high (0, 2, 8 and 9) range in Exp. 1 (Appendix 3) but these distinctions were not as clear in Exp. 2. Despite root damage from the fungus and perhaps the Benomyl, families displayed significant differences in their R:S ratios, but the order from high to low differed from Exp. 1 and there was not a significant
Fig. 1. Treatment effects on C:N ratio, biomass and R:S ratio in the N-source experiment. Each bar represents the mean of approximately 160 seedlings ± SEM. The SEM is based on the variance between the two replicates. (a) In Exp. 1, treatments were significantly different for biomass, $\alpha(2)=0.05$. (b) In Exp. 2, treatments were not significantly different in all variables.
Fig. 2. Relationship between total biomass and NUE in the N-source experiment. Symbols represent the average of 16 seedlings for each family within each treatment. The numbers beside each point represent the family. a). In Exp.1, the correlation coefficients are not significant within any of the treatments. In the NH$_4$NO$_3$ treatment, r= 0.30271 (P< 0.1945); in the NO$_3^-$ treatment, r= 0.06412 (P< 0.7883), and in the NH$_4^+$ treatment, r= 0.1513 (P< 0.5243). When all seedlings are combined, r= -0.58195 (P<0.007). (b). In Exp.2 the correlation coefficients were not significant within any of the treatments. In the NH$_4$NO$_3$ treatment, r= 0.12818 (P< 0.5902); in the NO$_3^-$ treatment, r= 0.04967 (P< 0.8352) and in the NH$_4^+$ treatment, r= 0.02324 (P< 0.9225). When all seedlings are combined, r= -0.63648 (P<0.0002).
family correlation between any of the treatments for this trait (Appendix 5, Table 1).

C. NUE (C:N ratio)

Mean treatment C:N values were not significantly different in both Exp. 1 and 2 (Appendix 2, Table 3). The graphical representation of treatment effects on C:N ratio shown in Figures 1a and b and 2a and b, indicated that treatment differences were significant. Pseudo-F tests did not confirm this difference possibly because of the small number of replicates and the significant treatment x replicate interaction. Two sample t-tests were done on pairs of treatments and the only significantly different pair was NO₃⁻ and NH₄⁺ in Exp. 1 (data not shown).

Families displayed significantly different C:N ratios, but there was no interaction between family and treatment (Appendix 2, Table 3). The range of family mean C:N values for each treatment was somewhat narrower for Exp. 1 (Exp. 1, 31.16- 35.79, Exp. 2, 29.77- 36.45). Multiple comparison tests showed that the ranking of families between the two experiments differed, but that each had only two clusters of family means. In Exp. 1, the higher NUE cluster contained families 1, 3, 7 and 10 and in Exp. 2 the higher NUE cluster contained families 0, 1, 2, 6, 7, 9 and 10 (Appendix 3).

D. Gas exchange measurements

Despite small sample sizes and large standard errors, there were significant treatment and family effects in certain gas exchange parameters (Fig. 3) (Appendix 4). Treatment and family differences were primarily expected in assimilation rate (A), yet this was the only factor to not show any significant differences. Treatment significantly affected stomatal conductance (gs), evapotranspiration (E) and the integrative factor Cᵢ/Cₐ, in relatively similar ways. gs, E and Cᵢ/Cₐ were highest in the NH₄⁺ treatment and lower in the NH₄NO₃ and NO₃⁻ treatments (Fig. 3b,c and d). Scheffe’s multiple comparison test indicated a significant difference between the NH₄⁺ and NH₄NO₃ treatments for gs, E and Cᵢ/Cₐ. Of these three variables, a treatment x family interaction only occurred in gs. This was evident in Fig. 3b where family 3 had the highest gs in the NH₄⁺ treatment, but dropped to almost equal to the others in the NO₃⁻ treatment. This trend was also seen in A and E, but was not nearly as pronounced.
Fig. 3. Treatment and family effects on measured gas exchange parameters in the N-source experiment. Numbers written above each bar in (a) represent the sample size and are the same for each parameter. Each of the bars represents the mean family value for (a) CO₂ assimilation rate, A, (b) stomatal conductance, gₛ, (c) evapotranspiration rate, E, and (d) the ratio of internal CO₂ concentration to ambient CO₂ concentration, Cᵢ/Cₐ. Treatments differ significantly for E (P < 0.0255), gₛ (P < 0.0509) and Cᵢ/Cₐ (P < 0.0501). Family differences are significant for E (P < 0.0231), gₛ (P < 0.0101) and Cᵢ/Cₐ (P < 0.0284). The treatment x family interaction was significant for gₛ (P < 0.0179).
Family differences were significant in gₐ, E and Cᵢ/Cₐ presumably because of the above mentioned influence of family 3. This influence was most evident in gₐ, as seen both in Fig. 3b and by Scheffe's multiple comparison test (Appendix 4), and became less pronounced in E, Cᵢ/Cₐ and A. Between treatments, the difference in families was most clear in the NH₄⁺ treatment and became more diffuse in the NH₄NO₃ and NO₃⁻ treatments.

E. δ¹³C

The different sources of N did not affect the mean δ¹³C of the seedlings (Appendix 2, Table 4). The spread of mean family δ¹³C values for all three treatments in the N-source experiment very nearly overlapped one another in both Exp. 1 and 2 (Fig. 4). δ¹³C values of seedlings in Exp.1 ranged from -28.78 to -31.42 %o and each of the NO₃⁻ and NH₄NO₃ boxes was a true replicate of its twin (i.e. family δ¹³C values in the replicates of the NO₃⁻ and NH₄NO₃ boxes were significantly correlated). Families in the replicates of the NH₄⁺ treatment ranked differently in each of the boxes and therefore replicates were not correlated. This did not affect the significance of the Error I term. Treatment replicates of Exp. 2 were all highly correlated (α(2) =0.05), but the δ¹³C range of the two NH₄⁺ tubs differed enough to cause a significant treatment by replicate interaction.

Families displayed significant variation in δ¹³C in both trials, but without a treatment effect, there was no treatment x family interaction. Family correlations between treatments were significant in Exp. 1, but in Exp. 2 were only significant in the NO₃⁻ versus NH₄NO₃ treatments (Appendix 5, Table 1). Family 0 stood out as having the highest δ¹³C regardless of treatment or fungal infection (Fig. 4). Removing this family from each of the treatments, reduced the range of δ¹³C values by almost half. The remaining families clustered, but continued to follow a similar ranking between treatments in Exp. 1 whereby families 3, 9 and 10 dominated the lower end of the spectrum. Endpoints of family 0 (least negative δ¹³C) and 3 (most negative δ¹³C in two of three treatments) held for Exp. 2 as well, but the order of the remaining families became less distinct (Fig. 4b). For instance, family 6 dropped down to a more negative value in all treatments and family 10 moved toward the middle of the range for all treatments in Exp. 2.
Fig. 4. Family $\delta^{13}$C rank in N-source experiment. Fig a and b represent Exp. 1 and Exp. 2, respectively. Each point represents the mean of 16 seedlings of a family within a treatment. Families are significantly different ($P < 0.0002$) for both experiments.
F. $\Delta\delta^{15}\text{N}$

Stable nitrogen isotope composition of the seedlings was the parameter least subject to measurement or method error. In Exp. 1, all factors were significant and in Exp. 2 all factors except the treatment x family interaction were significant (Appendix 2, Table 5). The mean seedling $\Delta\delta^{15}\text{N}$ was lighter than each of the respective salts and seedlings grown in the $\text{NO}_3^-$ treatment were more negative than both the $\text{NH}_4^+$ and $\text{NH}_4\text{NO}_3$ treatment seedlings. The differences between the $\delta^{15}\text{N}$ salt and plant $\delta^{15}\text{N}$ were: $\text{NH}_4^+ = 2.2005$, $1.033$, $\text{NH}_4\text{NO}_3 = 3.6795$, $3.0575$ and $\text{NO}_3^- = 4.09$, $3.49$ in Exp. 1 and 2, respectively. Family $\Delta\delta^{15}\text{N}$ values covered a similar range in the $\text{NH}_4^+$ and $\text{NH}_4\text{NO}_3$ treatments in both Exp. 1 and 2 (Fig. 5). In considering the $\text{NH}_4\text{NO}_3$ treatment data in Fig. 5b, it is important to note that the true $\delta^{15}\text{N}$ value of the source did not equal that of the $\text{NH}_4\text{NO}_3$ salt because of the additional $\text{NH}_4^+$ periodically added to maintain the $\text{NH}_4^+$/NO$_3^-$ ratio.

A significant Error I term indicates that replicates of the treatments were different from one another. In Exp. 1, mean family values in replicate boxes were highly correlated for both the $\text{NO}_3^-$ and $\text{NH}_4\text{NO}_3$ treatments, but not between replicates in the $\text{NH}_4^+$ treatment. This could be because the mean of one box was slightly more negative than the other (-1.236 %o vs. -1.705 %o) or because some families switched ranks between replicate tubs (families 1 and 3). A difference in replicate means did not occur in either of the other two treatments. In Exp. 2, almost the opposite is true and the correlation coefficient between replicate boxes is significant for the $\text{NH}_4^+$ treatment ($\alpha=0.05$), but not for either of the other two treatments. Differences between the means of the $\text{NO}_3^-$ and $\text{NH}_4\text{NO}_3$ replicates were 0.4 %o and 0.5 %o, whereas between $\text{NH}_4^+$ replicates it was only 0.2 %o. Changes in family rank were also more frequent in the $\text{NO}_3^-$ and $\text{NH}_4\text{NO}_3$ replicates than in the $\text{NH}_4^+$ replicates (data not shown).

Family rank by $\delta^{15}\text{N}$ value was also quite different between trials and within Exp. 1. In Exp. 1, there was a significant treatment x family interaction because of three substantial rank changes between treatments (families 2, 5 and 6) (Fig. 5a). In Exp. 2, there did not appear to be as many significant rank changes (treatment x family $P<0.5$), but the overall family order differed from Exp. 1. As well, the family correlations between treatments were not significant in either experiment (Appendix 5, Table 1). Family 0 consistently had one of the least negative $\delta^{15}\text{N}$ values, but all others changed between trials or treatments.
Fig. 5. Family $\Delta \delta^{15}$N rank in the N-source experiment. Fig. a and b represent Exp. 1 and Exp. 2, respectively. Each point represents the mean of 16 seedlings of a family within a treatment. Families are significantly different ($P < 0.005$) for Exp. 1 and not significant ($P < 0.0896$) for Exp. 2.
G. Biomass versus NUE and $\delta^{13}$C

Biomass and NUE were significantly negatively correlated between the three treatments in Exp. 1 and 2 (Fig. 2). Within treatments, there were no significant family correlations, but the correlation coefficient decreased from the NO$_3^-$ treatment to the NH$_4^+$ treatment. In addition, the distribution of biomasses covered a narrower range of C:N ratios. The families, however, were significantly different and maintained their relative rank or grouping through all three treatments. Only family 3 changed its rank from a relatively high biomass and high C:N ratio in the NO$_3^-$ and NH$_4$NO$_3$ treatments to a relatively low biomass in the NH$_4^+$ treatment.

Family correlations in Exp. 2 did not play out as clearly. There were no observable trends within the treatments. Family grouping was similar to Exp. 1, whereby families 1 and 10 represented the high end of the C:N range in both the NO$_3^-$ and NH$_4$NO$_3$ treatments and family 8 represented the lower end.

The expected positive correlation between biomass and $\delta^{13}$C on either an environmental or family basis was not seen in this experiment (Fig. 6). In both Exp. 1 and 2 the correlations appeared to be heavily influenced by the presence of family 0, but when this family was removed from the equation, the correlation coefficient did not increase and the slope of the line did not change. When all data were combined, a positive environmental correlation between biomass and WUE was still not evident.

There were, however, family patterns. Families 0 and 3 tended to dominate the extreme values in $\delta^{13}$C and controlled the correlation coefficient based on their biomasses relative to the other families in the treatment. All other families clustered in the middle of the range of $\delta^{13}$C values and served as the fulcrum for the balance created by the changing biomasses of families 3 and 0 in the NO$_3^-$ and NH$_4^+$ treatments. Families 10 and 1 tended to have the highest biomasses of the group, but were consistently in the middle to low end of the range of $\delta^{13}$C.

H. R:S ratio versus NUE and $\delta^{13}$C

R:S ratio correlated more significantly with NUE than with WUE. For both experiments, there was a significant positive correlation between R:S ratio and NUE (Exp. 1 $r = 0.6736$, $P<0.001$, Exp. 2 $r = 0.5227$, $P<0.003$) when all treatments were combined.
Fig. 6. Relationship between total biomass and δ¹³C in the N-source experiment. Symbols represent the average of 16 seedlings for each family within each treatment. The numbers beside each point represent the family. (a) In Exp. 1, the correlation coefficients are not significant within any of the treatments. In the NH₄NO₃ treatment, r= -0.16944 (P< 0.4751); in the NO₃⁻ treatment, r= -0.38651 (P< 0.0923) and in the NH₄⁺ treatment, r= -0.13682 (P< 0.5652). When all seedlings are combined, r= -0.16475 (P< 0.3843). (b) In Exp. 2, the correlation coefficients were not significant within any of the treatments. In the NH₄NO₃ treatment, r= 0.06821 (P< 0.7751); in the NO₃⁻ treatment, r= -0.06906 (P< 0.7723) and in the NH₄⁺ treatment, r= 0.00887 (P< 0.9704). When all seedlings are combined, r= 0.33579 (P< 0.0697).
When treatments were separated, the R:S ratio and NUE were negatively correlated for NH$_4^+$ and NH$_4$NO$_3$ treatments in both experiments (Exp. 1 $r = -0.4423$ and -0.457, P< 0.0509 and 0.0428, Exp. 2 $r = -0.7134$ and -0.6777, P< 0.0004 and 0.003, NH$_4^+$ and NH$_4$NO$_3$ treatments respectively). The distribution of the families along the regression line was very similar to what was obtained for biomass versus NUE (Fig. 2). Families with a high biomass and high NUE tended to have a low R:S ratio. There was no significant correlation between R:S ratio and $\delta^{13}$C for either experiment when treatments were combined or treated separately (data not shown).

I. $\delta^{13}$C versus NUE

A significant plastic trade-off between $\delta^{13}$C and NUE was not observed in either experiment (Fig. 7a and b). The six points on each figure represented the mean for all families in that treatment and did not indicate any trade-off. What the points indicated was the variability between replicates of each treatment. Another way of visualizing a plastic trade-off would be to average the replicates within each treatment for each family and then to plot $\delta^{13}$C against NUE for all 10 families in each treatment. The 10 regression lines should be parallel and have a negative slope when the independent variable is NUE. As NUE increases with the change in treatments (from NH$_4^+$ to NO$_3^-$), $\delta^{13}$C would decrease. Instead, when these data were plotted, the lines followed no particular pattern, some indicating a positive correlation, others indicating a negative correlation and still others presenting no correlation at all (data not shown).

When the mean values for all treatments within a family were plotted to show a family trade-off between the two traits, again there was no significant correlation (Fig. 7c and d). Families which had a high $\delta^{13}$C did not necessarily have a low NUE when averaged across treatments. Although there was genetic control of both of these traits, there was no inherent trade-off.

J. Biomass versus $\Delta\delta^{15}$N

Correlations between biomass and $\Delta\delta^{15}$N were inconsistent (Fig. 8). The environmental correlations for both experiments were significant and positive, but the family correlations varied. In Exp.1, the only significant family correlation with a positive slope was in the NH$_4^+$ treatment. The family correlation in the NO$_3^-$ treatment was significant as well, but had a
Fig. 7. Correlations between $\delta^{13}$C and NUE on environmental (a and b) family bases (c and d) in the N-source experiment. Figures (a) and (b) represent the environmental correlation for Exp.1 and Exp.2, respectively. Each of the points represents the mean of all seedlings in each of the replicates of the three treatments (80 seedlings). Neither of these correlations is significant: Exp.1 $r = -0.1109$ ($P < 0.5598$), Exp.2 $r = -0.3081$ ($P < 0.0977$). Figures (c) and (d) represent the family correlations for Exp.1 and Exp.2. Each of the points represents the mean of all seedlings of that family in all treatments (48 seedlings). Neither of these correlations is significant: Exp.1 $r = -0.2698$ ($P < 0.4508$), Exp.2 $r = -0.2104$ ($P < 0.5596$).
Fig. 8. Relationship between total biomass and Δδ¹⁵N in the N-source experiment. Symbols represent the average of 16 seedlings for each family within each treatment. The numbers beside each point represent the family. (a) In Exp. 1, the correlation coefficients are significant only for the NH₄⁺ and NO₃⁻ treatments and are indicated by the inserted line. In the NH₄NO₃ treatment, r= -0.203 (P< 0.3907); in the NO₃⁻ treatment, r= -0.4478 (P< 0.0477) and in the NH₄⁺ treatment, r= 0.6468 (P< 0.0021). (b) In Exp. 2, the only significant correlation coefficient is for the NH₄⁺ treatment and is indicated by the inserted line. In the NH₄NO₃ treatment, r= 0.06274 (P< 0.7927); in the NO₃⁻ treatment, r= -0.03884 (P<0.8709) and in the NH₄⁺ treatment, r= 0.71299 (P< 0.0004).
negative slope. In Exp. 2, the family correlations in the NH$_4^+$ treatment was significant and the slope was positive. In both experiments, the range of family $\Delta\delta^{15}$N values in the NH$_4^+$ and NH$_4$NO$_3$ treatments seemed less negative than the NO$_3^-$ treatment. Within Exp. 1, the range of $\Delta\delta^{15}$N values in the NH$_4^+$ and NH$_4$NO$_3$ treatments looked similar and families 2 and 6 in the NH$_4^+$ treatment and family 0 in the NH$_4$NO$_3$ treatment appeared to determine the slope of the line.

K. NUE versus $\Delta\delta^{15}$N

On an environmental basis, NUE and $\Delta\delta^{15}$N were significantly negatively correlated for both N-source experiments. The regression line was determined primarily by the three clusters of ten families in each treatment (Fig. 9). This negative correlation for both experiments was the only significant correlation between these two traits. On a family basis, correlations between the two traits within treatments were non-existent. Consistency in family ranking regarding the two traits was also difficult to determine.

L. $\delta^{13}$C versus $\Delta\delta^{15}$N

The environmental correlation between $\delta^{13}$C and $\Delta\delta^{15}$N was significant only in Exp. 2 (Fig. 10b). Of six possible family correlations in the two experiments, only one was significant at the $\alpha(2)=0.0124$ level and a second was significant at the $\alpha(2)=0.0641$ level (Exp. 1, NH$_4$NO$_3$ and NO$_3^-$ treatments, respectively). Although the NH$_4^+$ treatment in Exp.1 had a positive slope, most data points occurred in a very narrow band on the x-axis. Without families 2 and 6 it would appear that there was almost no variation in $\Delta\delta^{15}$N values in the NH$_4^+$ treatment, but there was family variation in $\delta^{13}$C. The range of $\Delta\delta^{15}$N values also decreased in the NH$_4$NO$_3$ treatment, yet the correlation was still significant.

As in other figures representing stable isotope data, family 0 again had the least negative value in both isotopes. This difference was most pronounced in Exp.1 in the NO$_3^-$ and NH$_4$NO$_3$ treatments, but continued in the NH$_4^+$ treatment as well. Beyond this distinction, there were not any ranks that were common to all three treatments. Families tended to cluster in particular ranges for each of the traits, but this was confounded when the range narrowed as it did for $\Delta\delta^{15}$N in the NH$_4^+$ treatment (Fig. 10a).
Fig. 9. Relationship between NUE and Δδ¹⁵N in the N-source experiment. Symbols represent the average of 16 seedlings for each family within each treatment. The numbers beside each point represent the family. (a) In Exp. 1, the correlation coefficients are not significant within any of the treatments. In the NH₄NO₃ treatment, $r = -0.2029$ (P < 0.3908); in the NO₃⁻ treatment, $r = 0.09499$ (P < 0.6904) and in the NH₄⁺ treatment, $r = -0.06683$ (P < 0.7795). (b). In Exp. 2, the correlation coefficients are not significant within any of the treatments. In the NH₄NO₃ treatment, $r = 0.06189$ (P < 0.7955); in the NO₃⁻ treatment, $r = 0.09391$ (P < 0.6937) and in the NH₄⁺ treatment, $r = -0.0937$ (P < 0.6939).
Fig. 10. Relationship between $\delta^{13}C$ and $\Delta \delta^{15}N$ in the N-source experiment. Symbols represent the average of 16 seedlings for each family within each treatment. The numbers beside each point represent the family. (a) In Exp. 1, the correlation coefficients are significant in the NH$_4$NO$_3$ and NO$_3^-$ treatments and are indicated by the inserted lines. In the NH$_4$NO$_3$ treatment, $r = 0.54766$ ($P < 0.0124$); in the NO$_3^-$ treatment, $r = 0.42158$ ($P < 0.0641$) and in the NH$_4^+$ treatment, $r = 0.09293$ ($P < 0.6968$). When all seedlings are combined, $r = 0.08481$ ($P < 0.6559$). (b) In Exp. 2, the correlation coefficients are not significant within any of the treatments. In the NH$_4$NO$_3$ treatment, $r = 0.31691$ ($P < 0.1734$); in the NO$_3^-$ treatment, $r = 0.28878$ ($P < 0.2169$) and in the NH$_4^+$ treatment, $r = -0.09735$ ($P < 0.6830$). When all seedlings are combined, $r = 0.40958$ ($P < 0.0246$).
In Exp. 2, the family patterns were different still. Families 3 and 6 were very light in carbon, but not necessarily so in nitrogen. Family 0 still had the least negative $\delta^{13}$C value for each treatment, but the same did not hold true for $\Delta\delta^{15}$N. The potential effects of the fungal infection could have caused more alterations to the nitrogen stable isotope ratio than to the carbon stable isotope ratio.

II. N- Supply Rate Experiment

A. Total biomass and allocation

Supplying ammonium in either a steady-state or draw-down method did not affect the total biomass of the seedlings. However within each treatment the family biomass means were significantly different and ranked similarly to Exp. 1 in the N-source experiment (Appendix 3). Again there was not a treatment x family interaction, but there was a treatment x replicate x family interaction indicating that families responded differently to the separate boxes because of unaccounted for factors (Appendix 2, Table 6).

After a log transformation of the R:S ratio in the N-supply rate experiment, observations met normality requirements and treatment, family and treatment x replicate x family effects were significant (Appendix 2, Table 7). Mean R:S ratio was higher in the draw-down treatment (draw-down = 0.436, steady-state = 0.261). A Tukey multiple comparison test showed that there were two clusters of R:S ratios, but family 3 was different from all other families and had the lowest R:S (Appendix 3).

B. NUE (C:N ratio)

For C:N ratio, N- supply rate experiment data were log transformed to meet normality requirements and treatment, treatment x replicate and family factors were all significant (Appendix 2, Table 8). Mean C:N ratio for the draw-down treatment (31.563) was higher than the steady-state treatment (21.743) (Fig.11), although the significant treatment x replicate interaction indicates that boxes were not true replicates of one another. Whether or not the replicate difference interacted with the family response could not be tested as it was for biomass and R:S ratio because of a lack of a sampling error. Families ranked similarly to Exp. 1 of the N-source experiments, strengthening the hypothesis that NUE is under genetic control (Figs 2 and 11) (Appendix 3)
Fig. 11. Relationship between total biomass and NUE in the N-supply rate experiment. Symbols represent the average of 16 seedlings for each family within each treatment. Open circles (○) denote the draw-down treatment and closed circles (●) denote the steady-state treatment. The numbers beside each point represent the family. Lines are included where the family correlation was significant. In the steady-state treatment, $r = 0.48010$ (P < 0.0322); in the draw-down treatment, $r = 0.67372$ (P < 0.0016).
C. Gas exchange measurements

Gas exchange measurements were more reliable and accurate for the N-supply rate experiment than for the N-source experiment. Sample size was slightly larger than it had been for the N-source experiment, yet there were fewer statistically significant measured variables. In fact, the only statistically significant variable was the treatment effect on the $C_i/C_a$ ratio (data not shown). Bar graph representations of the data showed similarities between the N-source and N-supply rate experiments in the family and treatment responses to treatments (Figs. 4a-d 12a-d).

D. $\delta^{13}C$

Treatment was a significant predictor of $\delta^{13}C$ when tested using a pseudo-F. The range of $\delta^{13}C$ values for each treatment was statistically similar (steady state $-29.89\%o$ to $-28.19\%o$, draw-down $-29.365\%o$ to $-27.735\%o$), but the mean for the draw-down treatment was less negative than the steady state treatment ($dd = -28.778\%o$ and $ss = -29.400\%o$) (Fig. 13). Error I was not significant and, unlike the N-source experiment, replicate boxes were highly correlated ($\alpha= 0.02$) (Appendix 2, Table 9).

Family was also a significant predictor of $\delta^{13}C$ when the treatment effect was removed. As in the N-source experiments, family 0 had the least negative $\delta^{13}C$ value and the others clustered in the lighter half of the treatment range. A multiple comparison test revealed that this family was the only one that was significantly different from any of the others. The closest followers in the steady state treatment were families 1, 2, 9 and 10, but this order changed in the draw-down treatment where families 2 and 10 switched to the lower range of $\delta^{13}C$ values. Families 3, 5 and 6 made up the more negative end of the range in the steady-state treatment, but only family 6 remained at that end in the draw-down treatment (Fig. 13). Although these families clustered in the lower half of the range, this variation in the ranks between treatments was represented in the significant treatment x family interaction.
Fig. 12. Treatment and family effects on measured gas exchange parameters in the N-supply rate experiment. Numbers written above each bar in (a) represent the sample size and are the same for each parameter. Each of the bars represents the mean family value for (a) CO₂ assimilation rate, $A$, (b) stomatal conductance, $g_s$, (c) evapotranspiration rate, $E$, and (d) the ratio of internal CO₂ concentration to ambient CO₂ concentration, $C_i/C_a$. The only significant factor was the treatment effect on $C_i/C_a$. 
Fig. 13. Relationship between total biomass and $\delta^{13}$C in the N-supply rate experiment. Symbols represent the average of 16 seedlings for each family within each treatment. Open circles (o) denote the draw-down treatment and closed circles (●) denote the steady-state treatment. The numbers beside each point represent the family. In the steady-state treatment, $r = 0.00616$ ($P < 0.0794$); in the draw-down treatment, $r = -0.28885$ ($P < 0.2304$). When all seedlings are combined, $r = -0.49403$ ($P < 0.0268$).
E. Δδ¹⁵N

Varying the supply rate of NH₄⁺ did not affect the Δδ¹⁵N of the seedlings, nor was there a treatment x replicate effect (Appendix 2, Table 10). Replicates within treatments were highly correlated (P< 0.01 for steady-state and P< 0.05 for draw-down), and therefore did not overshadow any potential treatment effects. Nine of the ten family Δδ¹⁵N values in the draw-down treatment were within the steady-state treatment range (Fig. 14). The variances of the two treatments were significantly different (α(2) =0.05, F> 4.03), indicating smaller family diversity in the draw-down treatment.

Most families became less negative in the draw-down treatment, but this variation could have been within the seedlings’ natural range or it could have been treatment induced. Considering that treatments were not significantly different for this trait, it is likely that the variation is within the normal genotypic range. Family Δδ¹⁵N values were significantly different (Appendix 2, Table 10). Ranking was relatively stable between treatments except for a few families. The Δδ¹⁵N value for family 0 varied little between treatments. The less negative end of the Δδ¹⁵N range also contained families 9 and 3 in the steady-state treatment and families 9 and 5 in the draw-down treatment. Although not very dramatic, some of these switches in ranking were very similar to what was seen in the N-source experiment between NH₄⁺ and NO₃⁻ (Fig. 8a and 14) (Appendix 3).

F. Biomass versus NUE and δ¹³C

The family correlation between biomass and NUE was significant and positive for both treatments (Fig.11). The spread of family mean C:N ratios in the steady-state treatment seemed to be primarily determined by four points (families 3, 8, 9, and 10), while all others clustered in one area. In the draw-down treatment, mean family values were more evenly dispersed.

For the most part, family mean ranking was consistent between the two treatments, but because of the cluster in the steady-state treatment this was difficult to determine. One notable switch was family 9 which changed from having a relatively high biomass and moderate C:N ratio in the steady-state treatment to one of the lower biomasses and C:N ratios in the draw-down treatment. Similar to Exp.1, families 3 and 10 had high biomasses and high C:N ratios in these treatments as well.
Fig. 14. Relationship between total biomass and $\Delta \delta^{15}$N in the N-supply rate experiment. Symbols represent the average of 16 seedlings for each family within each treatment. Open circles (o) denote the draw-down treatment and closed circles (●) denote the steady-state treatment. The numbers beside each point represent the family. Lines are included where the family correlation is significant. In the steady-state treatment, $r = 0.44921$ (P< 0.0469); in the draw-down treatment, $r = 0.0354$ (P< 0.8856). When all seedlings are combined, $r = 0.03994$ (P< 0.8672).
The correlation between biomass and $\delta^{13}C$ was significant on an environmental basis (Fig. 13), but on a family basis, neither regression was significant. Most families were less water-use efficient in the steady-state treatment. Family 0, as in Exp.1, seemed to heavily influence both correlations, but when this point was removed, neither coefficient was significant. Families maintained their rank for each trait within the treatments, except for family 2 which switched from a high WUE in the steady state treatment to a low WUE in the draw-down treatment.

G. $\delta^{13}C$ versus NUE

Unlike in the first two experiments, a significant plastic correlation between $\delta^{13}C$ and NUE occurred in these treatments ($r = 0.44109, P = 0.0516$) (Fig. 15a). Replicates of the steady-state treatment were not as similar as those of the draw-down treatment in their C:N ratio, but this difference was not large enough to mask a positive correlation between the traits. Although a trade-off between the two traits was expected, instead the environmental differences yielded seedlings with both a high NUE and less negative $\delta^{13}C$ (draw-down treatment) or a low NUE and more negative $\delta^{13}C$ (steady-state treatment).

A genetic negative correlation did not occur (Fig. 15b). Within the draw-down treatment there was a slight indication of a possible trade-off, but it was not significant ($r = -0.2986, P < 0.2143$). In the steady-state treatment, the cluster of families in a narrow range of C:N values created a flat line between the two traits. The extreme $\delta^{13}C$ values in family 0 were evident again in this experiment.

H. Biomass versus $\Delta\delta^{15}N$

Biomass and $\Delta\delta^{15}N$ were not significantly correlated across the two treatments. Within the steady-state treatment, the positive correlation was significant, but the correlation in the draw-down treatment was not (Fig. 14). Treatments appeared to have a substantial effect on the family rank for each trait. Fig. 14 indicated that the changes in families 3, 5, 9, and 10 between treatments were the greatest effectors for this switch from a positive to a negative correlation between treatments. Family 9 had a less negative $\Delta\delta^{15}N$ value, but was one of the largest
Fig. 15. Correlations between NUE and $\delta^{13}\text{C}$ on (a) environmental and (b) family bases in the N-supply rate experiment. Symbols represent the average of (a) all seedlings in each treatment box and (b) all eight seedlings of that family in all treatment boxes. Open circles (o) denote the draw-down treatment and closed circles (•) denote the steady-state treatment. The numbers beside each point represent the family. Lines are included where the correlation is significant. (a) The correlation coefficient using both treatments is $r = 0.44109 \ (P< 0.0516)$. (b) In the steady-state treatment, $r = 0.04576 \ (P< 0.8481)$ and in the draw-down treatment, $r = -0.29858 \ (P< 0.2143)$. 
families in the steady-state treatment and one of the smallest families in the draw-down treatment. Family 5 maintained its biomass ranking between treatments, but changed its isotope ranking from high to low. Families 10 and 3 also maintained their biomass rankings, but changed from being very heavy in the steady-state treatment to being very light in the draw-down treatment. Family 0 which always stood out as being very heavy, both in $^{13}$C and $^{15}$N, again was very heavy in this experiment, but also had a very low biomass in both treatments.

I. NUE versus $\Delta^{15}$N

C:N ratio and $\Delta^{15}$N were significantly correlated on an environmental basis but not within either of the treatments (Fig. 16). The positive environmental correlation was significant at the $P < 0.0697$ level, indicating that treatments which yield lower nitrogen contents are also isotopically heavier. The steady-state treatment showed absolutely no correlation because of the consistency of family C:N ratios within the treatment. Fig. 16 indicated that the correlation in the draw-down treatment was opposite that of the environmental correlation and families which had a high NUE had a relatively greater proportion of the lighter N isotope.

J. $\delta^{13}$C versus $\Delta^{15}$N

There was a positive relationship between $\delta^{13}$C and $\Delta^{15}$N across and within treatments. Between treatments the range of $\Delta^{15}$N values was broader for the steady-state treatment (variances were significantly different). Fig. 17 indicated that families grown with a steady-state supply of NH$_4^+$ spread out more evenly in both $\delta^{13}$C and $\Delta^{15}$N values, whereas families repeatedly starved of NH$_4^+$ tended to cluster in a more narrow range of $\Delta^{15}$N values. Only families 0, 5, 6 and 9 were separate from the group.

Most families became heavier in both C and N stable isotopes when grown under the draw-down conditions. Although the values became more positive, the magnitude of this change was different for each trait for each seedling. Families 0, 2, 3, and 10 did not follow this same pattern. Family 2 became lighter in its carbon isotope composition. Families 0, 3 and 10 became heavier in C but lighter in N. This backwards movement of the families reduced the $\Delta^{15}$N range in the draw-down treatment.
Fig. 16. Relationship between C:N ratio and Δδ¹⁵N in the N-supply rate experiment. Symbols represent the average of 16 seedlings for each family within each treatment. Open circles (o) denote the draw-down treatment and closed circles (●) denote the steady-state treatment. The numbers beside each point represent the family. In the steady-state treatment, r = 0.1115 (P<0.6399); in the draw-down treatment, r = -0.3849 (P<0.1036). When all seedlings are combined, r = 0.4139 (P<0.0697).
Fig. 17. Relationship between $\delta^{13}$C and $\Delta \delta^{15}$N in the N-supply rate experiment. Symbols represent the average of 16 seedlings for each family within each treatment. Open circles (o) denote the draw-down treatment and closed circles (●) denote the steady-state treatment. The numbers beside each point represent the family. Lines are included where the family correlation is significant. In the steady-state treatment, $r = 0.4429$ ($P < 0.0505$); in the draw-down treatment, $r = -0.4199$ ($P < 0.0735$). When all seedlings are combined, $r = 0.5673$ ($P < 0.0091$).
III. $^{15}$N Depletion Experiment

When the concentration of $\text{NH}_4^+$ was calculated from the measured $\mu$g of N found on each of the discs it frequently differed by sometimes twice as much from the molarity calculated using the phenolhypochlorite assay. The differences were greatest at the extremes of the range, concentrations greater than 200 $\mu$M or less than 20 $\mu$M. Although the differences between expected molarity and actual molarity at the extremes may have been related to the concentration procedure using the flash evaporator, it is more likely that these concentrations were beyond limits of accurate detection using the assay. The concentrations used in Fig. 18 were calculated from the elemental analyzer values divided by the sample volume.

Fig. 18 indicates that the media $\delta^{15}$N became more positive as $\text{NH}_4^+$ was removed from the solution in the draw-down treatment. In Fig. 18b, $D$, as calculated in equation 7, is plotted over the range of concentrations between sampling periods. The regression through the means of each of the concentration ranges is linear and significant.

In the steady-state treatment, the data was not as conclusive and it appeared that the media was becoming heavier although the concentration was staying the same. This would be expected for a certain amount of time, after which point the proportion of $\delta^{15}$N in the hydroponics media should plateau to equal the discrimination against it. Because the steady-state treatment was only sampled on two subsequent days, it is unknown whether the $\delta^{15}$N of the media ever reached this plateau (Box c $\delta^{15}$N = 23.19 %o and 19.71 %o, Box d $\delta^{15}$N = 6.11 %o and 14.44 %o, on Sept. 30 and Oct. 1, respectively).
Fig. 18. $^{15}$N depletion from hydroponics boxes. (a) The 13 points represent 3-4 samples taken from the two replicate boxes in the draw-down treatment (c and d) on two subsequent days (Sept. 30 and Oct. 1). The regression line is significant at the $\alpha(2) = 0.05$, $R^2 = 0.9628$. (b) Relationship between discrimination ($D$) and $[\text{NH}_4^+]$ in the boxes. Each of the lines represents the discrimination that occurred between the two points of sampling. The regression is significant at the $\alpha(2) = 0.05$, $R^2 = 0.497$. 
I. N- Source Experiments

A. Effects on individual traits

1. Environmental and genetic control of morphological traits

Typically in conifers, and frequently in plants growing on acidic soils, ammonium fertilization yields a higher biomass than nitrate fertilization (van den Driessche, 1971; Etter, 1972; Krajina et al., 1973; Bigg and Daniel, 1978; Scheromm et al., 1988; Marschner et al., 1991; Bedell et al., 1999), although this difference can often be quite small. Since the 1970’s, researchers have tried to distinguish what causes this difference in yield. Studies on uptake have shown that NH$_4^+$ is taken up much more readily than NO$_3^-$ (Kronzucker et al., 1997), but whether or not greater biomass follows from uptake or if there are other physiological effects on whole plant metabolism associated with N-preference has not been clarified. Differentiating between uptake, assimilation and allocation and the effects of N-demand on each of these cannot be done by simply looking at biomass. Growth response in conjunction with measures of sink strength and allocation, such as photosynthetic rate, C:N ratio or R:S ratio, can help to elucidate the mechanism for greater biomass.

The results of these experiments are similar to previous studies. Highest growth yield was achieved in the NH$_4^+$ treatments, followed closely by the NH$_4$NO$_3$ treatment and then the NO$_3^-$ treatment (Fig. 1). In the NH$_4$NO$_3$ treatments, NH$_4^+$ was depleted on a daily basis despite increasing pump rates and manual addition of NH$_4^+$. Conversely, the NO$_3^-$ concentration continued to increase as NH$_4$NO$_3$ was pumped into the hydroponics boxes. By the end of a one week period, the NO$_3^-$ concentration could reach over 100 μM (two times the intended 50 μM), yet NH$_4^+$ was still being depleted. This indicates either NH$_4^+$ inhibition of NO$_3^-$ uptake and/or an uptake preference for NH$_4^+$ (Gessler et al., 1998; Kronzucker et al., 1999). Distinguishing between these two possibilities is difficult without knowing the uptake rates of each of the ions. Kamminga-van Wijk and Prins (1993) presented more concrete evidence of NO$_3^-$ inhibition in Douglas-fir by calculating the change in V$_{\text{max}}$ and K$_m$ for NO$_3^-$ when supplied alone or in an NH$_4$NO$_3$ solution. Under NO$_3^-$ nutrition, the K$_m$ and the V$_{\text{max}}$ were 17 μM and 5 μmol/g root dry weight, respectively. In an NH$_4$NO$_3$ solution, uptake decreased so much that the K$_m$ and the V$_{\text{max}}$ could not be calculated (Kamminga-van Wijk et al., 1993). Gessler et al. (1998) found that...
inhibition of NO$_3^-$ uptake in spruce (*Picea abies*) and beech (*Fagus sylvatica*) trees correlated with an enhancement of the concentration of primary amino compounds involved in NH$_4^+$ assimilation (Glu, Asp and Gln). They did not measure the K$_m$ and V$_{max}$ of NO$_3^-$ uptake of seedlings fed both NO$_3^-$ and NH$_4$NO$_3$ solutions and have presumed that decreased uptake (as compared to NH$_4^+$ uptake) is due to inhibition and NH$_4^+$ preference. These two cases present evidence of NO$_3^-$ uptake inhibition, but whether or not it occurred in my experiments is unknown.

In Exp. 2, biomasses were again similar for the NH$_4^+$ and NH$_4$NO$_3$ treatments, but all biomasses appeared smaller than in Exp.1. Presumably this reflects the damage of either the fungus or the fungicide. Fungal effects could have been through the prevention of nutrient uptake by mucilage covering the roots, by utilization of carbohydrates produced by the plant or by simply damaging plant roots. Benomyl, the fungicide used, also could have inhibited growth through side effects on the plant. The chlorophyll fluorescence data indicate that these plants were stressed. Possibly because of these effects, there was no significant difference between the mean biomass in each of the treatments (Fig.1b).

Biomass allocation to subterranean versus aerial parts of the plant has also been attributed to differing types of nutrient stress. When plants are nitrogen or water limited, a larger proportion of the net carbon production is apportioned to root growth (Chapin, 1980; Birk et al., 1986; Bailey, 1999; Traore and Maranville, 1999). This increases the root surface area for absorption and allows roots to tap into new nutrient sources. Unlike biomass, mean R:S ratio was not significantly different in each of the treatments. Although Fig.1 indicates that R:S ratio was highest in the NO$_3^-$ treatment and lowest in the NH$_4^+$ treatment, statistical limitations such as low degrees of freedom and dissimilarity of the replicates may have prevented detection of significant differences. The implication of treatment differences in R:S ratio is that NO$_3^-$ fertilization resulted in a certain level of N limitation. Because the seedlings were not able to take up as much nitrogen, proportionally more biomass was allocated to root growth and less to leaf area. Increased allocation to root growth can also cause an increase in respiration as compared to photosynthesis (Ibrahim et al., 1998). This increase in respiration combined with less leaf area for photosynthate production could lead to a decreased biomass.

Genotypic control of plant response to nutrition has been noted for various species since the 1930’s: multiple species survey (Harvey, 1939), pine (Li et al., 1991), rice (Tirol-Padre et
al., 1996), sorghum (Traore et al., 1999) and peanut (Virgona et al., 1990). It is not surprising that the ten white spruce families used in these experiments showed genetic control of biomass and R:S ratio in each of the three N sources (Fig. 2). The consistency of family rank between treatments and the lack of a g x e interaction indicates that treatments did not affect the families differently. If the treatment effects on biomass were primarily through uptake capacity of NO$_3^-$ and NH$_4^+$, consistency in family ranking suggests that genetic control is not independent for the two N-sources. The implication is that families with enhanced ability to acquire NH$_4^+$ are also better at acquiring NO$_3^-$ and genotypic control of uptake and utilization capacity (where utilization capacity includes N demand and allocation) would apply to both N-sources. Alternatively, the consistency of rank and lack of a g x e interaction could indicate that physiological processes independent of nutrition are more important in determining growth rate differences.

2. Environmental and genetic control of NUE

Trends in plant NUE in response to treatment were as expected based on reported differences in N-uptake between N sources (Kronzucker et al., 1996). Although it appeared as though the mean C:N ratio was highest in the NO$_3^-$ treatment and lowest in the NH$_4^+$ treatment, there was no significant treatment effect (Fig. 1). This was likely due to statistical limitations caused by the low number of replicates. Treatment effects were calculated using a pseudo-F test which removes Error 1, the treatment x family interaction and Error 2, but reduces the degrees of freedom to 2/3. The low degrees of freedom decrease the likelihood of finding a significant difference between treatments. In addition, the significant replicate effect, although it was removed from the statistical test, produced differences that may have overshadowed treatment effects in this experiment. Statistical reasons may explain the lack of a significant treatment effect, but there could also be distinct physiological effects of each of the N-sources that may alter the NUE.

Outside of the NH$_4^+$ preference indicated in these experiments, there may have been differing physiological responses to the two N-sources. Evaluating whether or not N-source caused significant differences in assimilation or allocation may allow treatment effects to be simplified to high and low N. NUE can be altered by changing either C or N quantities in the plant. N-source could potentially affect either of these two parameters through 1) internal pH
changes, 2) the bioenergetic costs of the NO$_3^-$ reduction steps or 3) the regulation of the associated assimilating enzymes. Internal pH is regulated by the biochemical pH stat and through efflux of ions. The biochemical pH stat, which is likely to be most active when plants are well fertilized with one N form, adjusts the pH by the addition and removal of carboxylic acids (Raven et al., 1976). These experiments were designed to create N-stress situations, so it is unlikely that the two N-sources caused internal pH differences great enough to change the balance of carboxylic acids. More likely is the possibility that ions were fluxed into the hydroponics media. Marschner et al. (1991) used the increasing pH of the medium that supplied NH$_4$NO$_3$ fertilizer to the roots of Norway spruce trees to indicate when seedlings switched from NH$_4^+$ uptake to NO$_3^-$ uptake. They proposed that soil pH changes could affect the uptake of other mineral ions, thereby causing a nutrient deficiency (Marschner et al., 1986; Marschner et al., 1991). The buffering capacity of the added CaCO$_3$ should have mediated any N-uptake inhibition caused by external pH in these experiments. Thus, internal pH changes associated with N-source should not have significantly affected either C or N levels in the seedlings.

Energetic costs of NO$_3^-$ reduction depend on the location of N assimilation, light availability, and NO$_3^-$ uptake capacity. In conifers, where the majority of NO$_3^-$ reduction occurs in the root, reductant and ATP for N assimilation are derived from mitochondrial respiration. Although this may decrease the net plant carbon gain per gram of nitrogen taken up (decreased NUE), it would only be a significant cost if seedlings were removing NO$_3^-$ rapidly from the medium (Smirnoff et al., 1985). At the low N-concentrations used in this study the proportional energetic costs of NO$_3^-$ assimilation would be balanced by N uptake limitations and total carbon cost would be no greater than for NH$_4^+$ assimilation. On a genotypic basis, however, families with a higher $A_{max}$ may be able to provide more energy for NO$_3^-$ reduction than families with a lower $A_{max}$. In this way, genotypic differences in NUE within treatments may be influenced by differences in $A_{max}$.

The effects of the two N sources on N assimilating enzymes were not investigated in this experiment. Published data are variable and are affected by light quantity, N-source and the distribution of assimilating activity between NR, the two isoforms of GS, and GDH (Vezina et al., 1988; Knoepp et al., 1993; Truax et al., 1994). Without having analyzed the enzyme activity in either of my treatments, it is difficult to say how differences in enzyme activity may have contributed to the lack of treatment differences in C:N ratio.
A fourth possibility, potentially unrelated to N-source, would be an increase in C proportionally greater than the treatment induced N increase. This could occur if an increase in gs increased A or if there was increased allocation of leaf N to photosynthesis away from structural or defense compounds (Fig. 19). In this scenario, a positive environmental correlation would be expected between NUE and biomass. This correlation was not found in the N-source experiments (Fig. 2).

Regarding each of the four possibilities mentioned, it would seem that differences in NUE (and subsequently biomass and R:S ratio) were dominated by differences in uptake capacity of NO$_3^-$ and NH$_4^+$ seen previously in white spruce. The lack of a significant treatment effect was most likely due to statistical limitations and the unaccounted for replicate effect. Once the nitrogen had entered the plant, it is assumed that assimilation of the two ions did not alter physiology other than by bringing about differences in tissue N concentration.

The assumptions regarding genetic control of NUE rely on the fact that these treatments achieved their desired effect, creating low N environments where the limiting growth factor was nitrogen. In the study after which this one is modeled (Livingston et al., 1999), C:N ratios of seedlings in both low and high N fertilization treatments were above 45. Not one of the family mean C:N ratios in any of the treatments in this experiment was above 45, but these were 4 month old seedlings rather than 2 year old seedlings. At two years, proportionally more carbon is invested in non-photosynthesizing structures which increases the apparent NUE (Raven and Farquhar, 1990). Despite a seemingly low C:N ratio, these seedlings appeared nitrogen stressed; i.e., the seedlings were chlorotic or developed red pigmentation. Based on these visual cues and that N was the most limiting nutrient supplied by a factor of approximately 10, it appeared that the low N-concentrations in the media produced the desired N-stress.

Genetic control of NUE, as seen with these seedlings, can be divided into four components: uptake capacity, assimilation capacity, allocation and growth-driven demand (Fig. 19). The effect of each of these components on measured parameters can be predicted if all other components are not limiting or do not vary (Table 2). For example, genotypes with a high uptake capacity should also have a high growth rate, low NUE, more positive $\delta^{13}$C and more negative $\delta^{15}$N. In reality, such predictions may not hold because of the relative contribution of the other three components and the changing prioritization of components of NUE with the environment. The table is presented as a framework to focus the discussion and summarize the
Fig. 19. Schematic diagram of components of NUE. Although the potential N sinks can create a demand for N, the primary N demand is through growth. This demand can positively affect assimilation and influx, and negatively affect efflux. The strength of the demand and its effects on efflux/influx, assimilation and allocation may alter whole plant NUE, biomass, R:S ratio, $\delta^{13}$C and $\delta^{15}$N.
Table 2. Expected genotype response in measured variables due to differences in the various components of NUE (uptake, assimilation, allocation and demand/ growth rate).

<table>
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<tr>
<th>Differences in N uptake capacity</th>
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<tbody>
<tr>
<td>low uptake capacity</td>
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<tr>
<td>low growth rate</td>
</tr>
<tr>
<td>high NUE</td>
</tr>
<tr>
<td>more –ve $\delta^{13}C$ (low WUE)</td>
</tr>
<tr>
<td>more +ve $\delta^{15}N$</td>
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<tr>
<th>Differences in N assimilation capacity</th>
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<tr>
<td>low assimilation capacity</td>
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<tr>
<td>low growth rate</td>
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<tr>
<td>low NUE</td>
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<tr>
<td>more –ve $\delta^{13}C$</td>
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<tr>
<td>more –ve $\delta^{15}N$</td>
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<thead>
<tr>
<th>Differences in N allocation at leaf level</th>
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<tbody>
<tr>
<td>low allocation to Ps</td>
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<tr>
<td>low growth rate</td>
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<tr>
<td>low NUE</td>
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<tr>
<td>more –ve $\delta^{13}C$</td>
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expected isolated effects of each of the components on measured parameters. Most genotypes exist between the extremes of high and low and likely vary in more than one component.

Rank maintenance of NUE indicates that control of NO$_3^-$ and NH$_4^+$ uptake is correlated, in which case treatment effects are reduced to high and low nitrogen. Unfortunately, there are no other genetic studies on NUE in conifers which test genetic control by placing genotypes in differing N source treatments. Most studies in conifers, and few in crop plants, that have investigated NUE using genotypes as a factor have used high and low levels of fertilization; pumpkin (Tan and Hogan, 1995), pine (Li et al., 1991), spruce (Livingston et al., 1999), rice (Tirol-Padre et al., 1996). Only in sorghum were differing N sources tested, and a g x e interaction was evident in two of four genotypes tested (Traore et al., 1999). A g x e interaction was not evident in these experiments, supporting the conclusions regarding control of the uptake carriers drawn from rank maintenance.

3. Environmental and genetic control of WUE

Neither of the two measures of water-use efficiency that were employed showed very clear effects of N-source. Treatments affected the instantaneous measure, C$_i$/C$_a$, but not the long-term measure, $\delta^{13}$C (Fig. 3d and 4). The absence of a treatment effect could be due to small degrees of freedom and the replicate effect, or it may be real. The results are surprising considering that in a previous study using both high and low N treatments and nine of the same families, these two measures of WUE were highly correlated and N fertilization significantly affected A and $\delta^{13}$C (Livingston et al., 1999). Other possible explanations for this discrepancy between the two studies are that treatments were not extreme enough to cause an effect on WUE or that there were unexpected environmental effects of hydroponics.

The NH$_4^+$ treatments were expected to affect gas exchange by increasing the rate of CO$_2$ fixation. The preference for NH$_4^+$ would provide more N for Rubisco and other proteins, thereby increasing A (Evans, 1989; Brown et al., 1996). This was not evident in the study possibly because at such low N-concentrations the treatments were not different enough to significantly alter A (Fig. 3). Patterson (1994) also did not find significant differences in A in white spruce when fertilized with high (5.6 mM) and low (0.6 mM) levels of N when seedlings were well-watered. Under droughted conditions, A was significantly reduced in the low-N treatment.
Watering, and perhaps more so, hydroponics, could alter the N-source effect through gs. If plants operate within a consistent range of Cj, then increases in the A\text{max} with N-fertilization should also increase the gs (Wong et al., 1979). In a study on sunflowers, Virgona and Farquhar (1996) found that N-limitation had greater effects on genotypic variation in A than gs. Toft et al. (1989) found the opposite in four species of cold desert shrubs and grasses. Fertilizing increased the quantity of Rubisco, but gs decreased and A was kept constant (Toft et al., 1989). In a third study that used hydroponics, fertilization increased A but not as much as gs (Ranjith and Meinzer, 1997). The results from the N-source experiments are more like the third example, gs increased with NH\textsubscript{4}\textsuperscript{+} fertilization, but A was constant (Fig. 3). This supports the hypothesis that hydroponics might create a situation different from the expected whereby gs overcame treatment effects on A\text{max}.

The effect of the fungus on the majority of the gas exchange data can also not be ignored. If the fungus and subsequent loss of roots or root function caused a perceived drought even though the plants were in hydroponics, this could have had a more substantial effect than either N-source. The range of Cj/Ca values was similar to that of the non-irrigated seedlings in Livingston et al. (1999) and the assimilation rates were similar to assimilation rates found in non-irrigated white spruce seedlings found in Patterson et al. (1997). The significantly lower gs in the NO\textsubscript{3}\textsuperscript{-} treatment may indicate a fungal preference for NO\textsubscript{3}\textsuperscript{-}. If the fungus grew better on a NO\textsubscript{3}\textsuperscript{-} substrate, then the seedlings in both the NH\textsubscript{4}NO\textsubscript{3} and NO\textsubscript{3}\textsuperscript{-} treatment boxes would be more droughted and have a lower gs than the NH\textsubscript{4}\textsuperscript{+} treatment (Fig. 3b). This would result in a relatively larger decrease in E than A, but also a decrease in Cj/Ca (Fig. 3c and d).

Family differences may also be a reflection of fungal tolerance or resistance rather than family response to intended treatments. Family means were not significantly different in A, but family 3 was significantly different from the others in both gs and E. This difference in gs in the NH\textsubscript{4}\textsuperscript{+} treatment may have been the sole reason for the treatment differences in these two parameters. Although it was not visually obvious, family 3 may not have been as infected as the others and because of an inherent NH\textsubscript{4}\textsuperscript{+} preference was healthier and suffered less damage from the fungus in treatments containing NH\textsubscript{4}\textsuperscript{+}. This interpretation is consistent with its exceptionally large biomass in the NH\textsubscript{4}\textsuperscript{+} treatment (Fig. 2b), and with the very negative δ\textsubscript{13}C values in all treatments (Fig. 4b). Family 3 could have perceived less drought and had higher gs because of a less limited water supply, which reduced both the instantaneous and long term WUE.
Similar to gas exchange, N-sources should not have differing effects on the C stable isotope composition of these plants other than by providing more or less N for photosynthetic machinery. The prediction was that the NH$_4^+$ preference in uptake would provide more nitrogen to the seedlings than the NO$_3^-$ treatment, causing a higher WUE. The statistical and physiological explanations used for lack of significance in treatment effects on C:N ratio can also be applied here. Statistically, the low degrees of freedom available for the pseudo-F test may have limited the detection of significance between treatments. In addition, the significant difference between replicates compounded the statistical limitations of the low degrees of freedom. Physiologically, the N concentration fed to the plants may have been too low for both N-sources to cause a treatment effect on $\delta^{13}$C. If the treatment N concentrations were higher, possibly the disparity in uptake capabilities for the two N-forms would cause a greater difference in C:N ratio and $\delta^{13}$C.

There was, however, a significant genetic effect on $\delta^{13}$C. The graph of family ranks (Fig. 4) indicates that most families maintained their rank through most treatments in both experiments. Families that were most water-use efficient in the NH$_4^+$ treatment were also the most water-use efficient in the NO$_3^-$ treatment. There were some rank changes between treatments, but these were not large enough to yield a significant interaction effect in the ANOVA. Maintenance of rank implies either coordinated genetic control of N uptake between N-sources, or that other processes not investigated in this study play a larger role in determining differences in WUE.

Based on the previous data (Livingston et al., 1999) and established theory (Farquhar et al., 1982), it was expected that gas exchange data and stable carbon isotope composition would be concurrent indicators of WUE. After completing both Exp. 1 and 2, it was apparent that correlation between $C_i/C_a$ and $\delta^{13}$C would be unlikely because of the small number of families used in gas exchange and the substantial weight of the fungal infected experiment on the data set. The strong influence of family 3 may have been the sole basis for treatment differences in $C_i/C_a$. When the other seven families were included in $\delta^{13}$C analysis, this influence may have been dampened and treatments appeared more similar. If the effect of the fungus interacted with treatment effects through a NO$_3^-$ preference, then the NO$_3^-$ treatment would yield the highest WUE (low $C_i/C_a$ and less negative $\delta^{13}$C). But when the fungus was not present, the NH$_4^+$
B. Effects of correlated traits

1. Biomass versus $\delta^{13}$C

Both positive and negative environmental correlations between biomass and $\delta^{13}$C can occur, depending on treatment effects. Typically, a positive correlation occurs when treatments cause greater variation in $A_{\text{max}}$ than $g_s$ (e.g. N treatments) (Sun et al., 1996; Virgona et al., 1996; Patterson et al., 1997; Livingston et al., 1999). The negative correlation occurs when $A_{\text{max}}$ varies less than $g_s$ (e.g. irrigation or elevation treatments) (Holowachuk, 1993; Zhang et al., 1994; Aitken et al., 1995; Sun et al., 1996). The intent in using hydroponics was to minimize drought effects on $g_s$ and vary $A_{\text{max}}$ by changing the N treatment. The lack of environmental correlation between biomass and $\delta^{13}$C (Fig. 6) indicates that either hydroponics did not achieve the goal of keeping $g_s$ relatively constant or that treatment differences were not as distinct as planned.

Based on the fact that there were no significant treatment effects on NUE, $A_{\text{max}}$ or $\delta^{13}$C, it would seem that the lack of correlation between biomass and $\delta^{13}$C was because of the similarity in treatments or dissimilarity of replicates.

Families were significantly different in both biomass and $\delta^{13}$C for both experiments, yet the two variables were not correlated within any treatment (Fig. 6). As with the environmental correlation, a positive genetic correlation between the two traits relies on genetic variation in $A_{\text{max}}$. This was not evident in the three families tested for gas exchange. Wong et al. (1979) proposed that within limits, $g_s$ is determined by $A_{\text{max}}$. This connection between $g_s$ and $A_{\text{max}}$ has appeared repeatedly when plants are grown in soil (Field et al., 1983; Sage et al., 1987; DeLucia et al., 1991; Meinzer et al., 1992), and in hydroponics (Ranjith et al., 1997). If fertilization directly affects stomatal control, in addition to its effect on $A_{\text{max}}$, family differences in $\delta^{13}$C may reflect stomatal responses. Genetic determination of stomatal sensitivity to N treatments could cause variation from the predicted positive correlation between biomass and $\delta^{13}$C. Alternatively, there may be an unaccounted for reaction to hydroponics that has altered the normal family correlation.
2. Biomass versus NUE

Correlations between biomass and NUE yielded predicted results on both environmental and family bases. The negative environmental correlation between biomass and NUE in both Exp. 1 and 2 (Fig. 2) shows that seedlings produced a higher biomass on treatments which provided higher internal N concentrations. If the two traits are negatively correlated, uptake preference has presumably determined NUE by increasing internal N more than C. If the correlation were positive, one N-source would have to result in greater C accumulation per unit N. This would only occur if the N-source caused allocation of proportionally more N to photosynthesis or other growth demand (Fig. 19).

Family correlations in Exp. 1 were positive but in Exp. 2, where the fungal influence may have altered uptake capacity for the families, correlations were not as clear. A positive correlation may indicate family differences in $A_{\text{max}}$ which cause a proportionally greater increase in C per unit N (Table 2). The strength of the correlation may be mediated by an N-source preference. Under the NO$_3^-$ treatment, efficient allocation to C assimilating proteins may be a priority. When N is plentiful, as in NH$_4^+$ fertilization, more N can be stored (e.g. luxury consumption) or allocated elsewhere and the correlation between NUE and biomass is not as clear. Thus, unlike the environmental correlation where N-source uptake preference determines the relationship, the genotypic correlation may depend on both uptake and utilization (Table 2).

3. NUE versus WUE

There are many studies that have shown an environmental trade-off between WUE and NUE (Field et al., 1983; Sage et al., 1987; Reich et al., 1989; DeLucia et al., 1991; van den Boogaard et al., 1995; Patterson et al., 1997; Livingston et al., 1999). The theory behind the trade-off is sound as long as certain correlative steps are satisfied. If one of those steps is not satisfied, then the trade-off is no longer apparent. The first required step is that fertilization will increase $A$ of the plants. There was no evidence that this occurred when the 10 families of white spruce were fed with varying forms of nitrogen in hydroponics (Fig. 3a). Without this correlation, it is difficult to discuss the effects of increased $A$ on the plastic WUE of the seedlings.

A second possibility, which would obviate any potential environmental trade-off, could occur in situations where one of the resources is so plentiful that its effect is null. For example,
in hydroponics, the plentiful supply of water may change the typical response of stomata to the environment. Abundant water may allow greater \( g_s \), permitting free exchange of carbon isotopes and \( \delta^{13}\text{C} \) would approach that of fractionation at Rubisco plus source \( \delta^{13}\text{C} \), or about -37 \(^\circ\text{o}\). This did not occur, indicating that there were stomatal limitations to carbon fixation. After conducting multiple experiments which altered \( g_s \) independently of \( A_{\text{max}} \), Wong et al. (1985) concluded that \( g_s \) may respond not to \( C_i \), but to a metabolite of photosynthesis (Wong et al., 1985). If this response was enhanced because of hydroponics, it may have contributed to the lack of correlation in Exp. 1 (Fig. 7a).

In Exp. 2, although it is not significant, the slope of the regression line is negative (Fig. 7b). If less negative \( \delta^{13}\text{C} \) values reflect drought imposed by the fungus or the Benomyl, despite being in hydroponics, then a decrease in \( g_s \) would contribute to the trade-off. The NH\(_4^+\) treatment would have provided more N for assimilation and therefore a higher WUE. The similar C:N ratios between the two experiments indicate that the seedlings were able to recover after the fungus had been killed, but the \( \delta^{13}\text{C} \) signature that had been developed was still present. The indication of a trade-off in this experiment implies that resource use efficiency trade-offs will occur only when the variation in environment incorporates both stresses. In Livingston et al. (1999), if the treatments are separated into irrigated and dryland, the negative correlation seen in the dryland treatments no longer exists within the irrigated treatment between nitrogen stressed and fertilized treatments.

A third factor to consider is the allocational differences that are incorporated when C:N ratio is used as the index of NUE. Under NH\(_4^+\) fertilization, luxury consumption would not contribute to improving the plastic WUE response. If luxury consumption is responsible for the decreased slope between biomass and NUE in the NH\(_4^+\) treatment (Fig. 2a), then a higher WUE would not be concomitant with this treatment. It may have been more informative to have used the instantaneous measure of NUE, \( A_{\text{max}}/N_{\text{leaf}} \) against the instantaneous measure of WUE, \( A/E \).

There was also not an inherent family trade-off between the two resource-use efficiencies. Although there is genotypic control of each of the traits, a genotypic trade-off between the two may not occur for various reasons. If families differ much in their allocational patterns both in biomass allocation and in allocation of N to particular processes other than photosynthesis, then a trade-off may not be visible (Table 2). Secondly, families may differ in their stomatal responses to environmental cues, and these differences may have greater influence.
over WUE than does internal N quantity. Genetic differences in gs were seen between family 3 and families 7 and 1 in the experiment infected by fungi but data is not available for healthy seedlings. This genetic difference may be a result of fungal resistance and not enhanced WUE, except that the low δ13C value for family 3 in all treatments supports high stomatal conductance and high Cj/Ca. Nonetheless, Amax was not significantly different for the three families, indicating that at the genetic level, multiple variables can confound the potential trade-off.

C. Effects on Δδ15N

1. Environmental and genetic effects on Δδ15N

The use of stable nitrogen isotopes at natural abundance levels as a tool to understand the uptake and metabolism of nitrogen is still a relatively new field. The data that has come from these experiments has shown that there is a possibility to use Δδ15N, in conjunction with other measures, to illustrate differences in N uptake and utilization as it relates to varying N sources and varying families. Of all traits or variables measured in these experiments, only Δδ15N showed significant treatment and family differences, as well as a significant g x e interaction. This indicates that it may be a reliable and sensitive variable to use when analyzing certain facets of N nutrition.

By removing the δ15N value of the salts from each of the seedling values, the remaining treatment effect is due only to differences in the physiological reaction to NO3− versus NH4+. One easy means to explain this difference would be to presume that the initial enzymes responsible for each type of N assimilation discriminate to a greater or lesser extent. Discrimination against 15N in the nitrate reductase reaction is 15 %o (Ledgard et al., 1985) and for glutamine synthetase (GS) the discrimination factor is 16.5 %o (Yoneyama et al., 1993). The 1%o difference between these two enzymes may not be significant and is much less than the differences between the NO3− and NH4+ treatments seen in this study. Therefore, enzymatic differences in discrimination can not account for the patterns observed.

A second possibility relates to influx and efflux analysis. Kronzucker et al. (1997) discovered through using radioisotope tracers that the storage capacity for NO3− in white spruce in both the cell-wall free space and the cytoplasm of the roots is five to eight fold lower than for NH4+. A small storage capacity implies that NO3− which is not immediately assimilated by NR is quickly effluxed back out of the root (Fig. 19). Knoepp et al. (1993), in studying assimilating
enzymes in western hemlock, realized that NR activity under NO$_3^-$ fertilization is less than 1/2000 of GS activity in either NO$_3^-$ or NH$_4^+$. At such low rates of assimilation, it is possible that NR is limiting N acquisition for the plant. NO$_3^-$ is taken into the root, but because NR is saturated, the internal NO$_3^-$ pool is able to remain closer to isotopic equilibrium with the outside pool (i.e. the lighter isotope is preferred whereas the heavier isotope has a better chance of efflux). Although NO$_3^-$ fertilization can be considered a stress treatment, it does not confer a heavier isotope signature because of the limitations of nitrate reductase activity and the inability to store large quantities of internal NO$_3^-$ prevent $^{15}$N accumulation.

The NH$_4^+$ and NH$_4$NO$_3$ treatments are similar in Exp. 1 because of more rapid uptake and assimilation of NH$_4^+$ versus NO$_3^-$ in the NH$_4$NO$_3$ treatment. Because NH$_4^+$ was consistently resupplied in the NH$_4$NO$_3$ treatment, the proportion of N assimilation as NO$_3^-$ was much smaller than it was in the NO$_3^-$ treatment. In Exp. 2, $\Delta\delta^{15}$N values from the NH$_4$NO$_3$ treatment are confounded by the fact that a 50:50 ratio of NH$_4^+$ to NO$_3^-$ was maintained by pipetting NH$_4^+$ into the treatment boxes to raise the NH$_4^+$ concentration without raising the NO$_3^-$ concentration. Because of the pipetting in of NH$_4^+$ salt, with a different $\delta^{15}$N value than the NH$_4$NO$_3$, an exact value for the source $\delta^{15}$N could not be computed (Fig.5).

The demonstration of genotypic differences in stable N isotope signature is quite novel. Genotypic control was first shown in barley under salt stress treatments (Handley et al., 1997) and has subsequently been shown with N and water stress treatments (Robinson et al., 2000). In Exp. 1, most of the 10 families maintained their approximate $\Delta\delta^{15}$N rank in the three different N-sources despite a few interactions with treatment (Fig. 5). The substantial rank change of families 2 and 9 may have contributed to the significant g x e interaction. Family differences were also apparent in Exp. 2, but the ranking was less clear. Again this could be a result of the fungal interaction with uptake capacity. Nonetheless, in both experiments it is presumed that the differences in $\Delta\delta^{15}$N were a result of family differences in the ratio of efflux to influx.

2. Correlations with biomass, NUE and $\delta^{13}$C

Proposed theories for explaining treatment and family effects are more meaningful if they support correlations between traits. The simplest trait to use to understand $\Delta\delta^{15}$N is biomass. The plastic response to N-source treatments was that bigger, NH$_4^+$ or NH$_4$NO$_3$ grown seedlings accumulated more of the heavy isotope (Fig. 8). Previously, the trade-off between biomass and
NUE was attributed to an N-source preference in uptake for NH$_4^+$, which in turn, provided more N for growth. N-source preference in uptake alone does not fully explain the correlation with $\Delta\delta^{15}$N. If the initial assimilating enzyme represents the sink for N, then NH$_4^+$ was not only taken up more quickly but was also assimilated more rapidly at a rate approaching uptake (i.e. efflux/influx would be lower) (Fig. 19). These two factors yielded seedlings with greater biomass and more positive $\Delta\delta^{15}$N values than seedlings in the NO$_3^-$ treatment.

The genetic correlation between biomass and $\Delta\delta^{15}$N was more variable than the environmental correlation. In Exp. 1 the negative correlation in the NO$_3^-$ treatment and the positive correlation in the NH$_4^+$ treatment were both significant (Fig. 8a). If differences in $\Delta\delta^{15}$N are determined more by N demand and assimilation than by uptake per se, then family correlations within treatments should all be positive. On the other hand, if uptake capacity is variably limiting, as it may be for NO$_3^-$, the efflux/influx ratio will be low and $\Delta\delta^{15}$N will correlate negatively with biomass (Table 2).

In both Exp. 1 and Exp. 2, the environmental correlation between the C:N ratio and $\Delta\delta^{15}$N was negative (Fig. 9). In the paper previously mentioned (see Literature Review IV.A) on the stress response of barley genotypes (Robinson et al., 2000), decreased $\delta^{15}$N from both drought and N-starvation was attributed to a loss of $^{15}$N-enriched organic N. If the NO$_3^-$ treatment created a higher degree of N-stress, then these results agree with Robinson et al. Alternatively, if the lighter stable isotope composition in the NO$_3^-$ treatment was a result of a lower uptake, storage and assimilation capacities, loss of organic N would not seem to be a major contributor to plant $\delta^{15}$N. Measuring the inorganic and organic stable isotope composition of N released from the plant would help in understanding whether NUE and $\Delta\delta^{15}$N are correlated through the exudation of organic N or through the efflux of unassimilated N caused by an imbalance between the uptake and assimilation of inorganic N.

A family correlation between NUE and $\Delta\delta^{15}$N was variable to non-existent (Fig. 9). This could be related to the four components of NUE and their effects on one another. If this is reduced to simply genetic control of uptake and assimilation capacity, genotypes could display four different phenotypes in response to fertilization. There could be genotypes that continue to competitively take up nitrogen at very low levels and assimilate all that is taken up. These plants would have a high NUE and low $^{15}$N/$^{14}$N ratio until N quantity of the nutrient solution becomes
scarce. At this point the enhanced uptake capacity will allow seedlings to continue taking up heavy N and the $\delta^{15}N$ value will approach that of the media. Alternatively a genotype could take up nitrogen well, but have a low demand for N. This would yield plants with $\delta^{15}N$ values more negative than the fertilizing solution because of the efflux of heavy inorganic N. The proportion of N that is effluxed and the demand for N will determine NUE. If a genotype has a low uptake capacity but a high assimilation capacity, then NUE would be high and $\delta^{15}N$ would always be near that of the media. Finally, if a genotype is relatively poor at both components, it will have a high NUE, but be very light because heavy N is still effluxed due to the low assimilation capacity. As N is removed from the medium, these genotypes will be slower to reach the $\delta^{15}N$ of the media because of low uptake capacity. Families that potentially represent each one of these scenarios were seen in the treatments. Such varied results could have caused the lack of family correlation between the two traits. In all cases, however, $\Delta\delta^{15}N$ would be expected to reflect efflux/influx ratio if this information were available.

Interestingly, there was a positive environmental correlation between $\delta^{13}C$ and $\Delta\delta^{15}N$ in Exp. 2 but not in Exp. 1 (Fig. 10). The plastic correlation may be a result of inadvertent drought stress due to root damage. Because of the root damage, treatments may have caused differences in $\delta^{13}C$ that would not normally have been apparent under hydroponics (Exp. 1). Root damage would also increase N demand relative to N uptake, reducing the efflux/influx ratio. A positive correlation between $\delta^{13}C$ and $\Delta\delta^{15}N$ would result.

Similar to both existing barley studies (Handley et al., 1997; Robinson et al., 2000), there was a positive family correlation between the two stable isotope measures, $\Delta\delta^{15}N$ and $\delta^{13}C$, in five of the six family regressions (Fig. 10). A physiological rationale for this is not clearly evident from the graphs. Of the five positive correlations, the two significant regressions are in Exp. 1 in the $\text{NO}_3^-$ and $\text{NH}_4\text{NO}_3$ treatments. If these two treatments represent higher efflux/influx ratios than the $\text{NH}_4^+$ treatment, the positive correlation between the stable isotopes could reflect sink strength or the capacity for N allocation to photosynthesis (Fig. 19). Families which had less negative $\Delta\delta^{15}N$ values because of low uptake capacity and high N-demand may have allocated proportionally more N to photosynthesis which would create a less negative $\delta^{13}C$ value. On the other hand, families which had relatively less of both C and N heavy isotopes in the $\text{NO}_3^-$ and $\text{NH}_4\text{NO}_3$ treatments may not be as efficient at allocating N to photosynthesis when
N quantities are low. When these same families are placed in the NH$_4^+$ treatment, where uptake is higher because of the N-source preference, less efflux of N and an increase in $A_{\text{max}}$ could cause both $\delta^{13}$C and $\Delta\delta^{15}$N to become less negative.

These scenarios are dependent on multiple physiological balances and, for the most part, are hypothetical. The underlying theory could be tested in different environments and with different species using $\delta^{15}$N in conjunction with other techniques (e.g. efflux analysis). In addition, it is important to assess in what form the heavy N is being lost.

II. N-supply rate experiment

After receiving results from the first and second experiments, a modification was made to the experimental design in order to acquire more information about environmentally and genetically controlled nitrogen isotope discrimination. Maintaining equal concentrations of NO$_3^-$ and NH$_4^+$ in the NH$_4$NO$_3$ treatments and maintaining equal concentrations of each ion in their own treatments proved to be very difficult, so it was proposed to eliminate the NO$_3^-$ and NH$_4$NO$_3$ treatments and use two treatments of NH$_4^+$. One treatment would maintain a steady state of NH$_4^+$ supply at 200 µM and the other treatment would begin at 200 µM and decrease down to approximately 0 µM, in order to test how different supply regimes would affect plastic and genetic control of N discrimination. I speculated, for example, that isotope enrichment during nutrient draw-down might separate families better able to acquire N at low concentrations. The same parameters were measured as before: biomass, R:S ratio, C:N ratio, $\delta^{13}$C and $\delta^{15}$N.

A. Effects on individual traits

1. Environmental and genetic control of morphological traits

On the whole, mean biomass was greater in this experiment than in the NH$_4^+$ treatment of the N-source experiment because of the higher concentration of NH$_4^+$ supplied. Significant effects of supply regime on biomass were not expected and did not occur. If the rate of uptake is fairly constant as the source N decreases (as reported by Clement, 1978), then biomass differences would only occur if the seedlings in the draw-down treatment were kept at 0 µM for extended periods of time. In this experiment, once the NH$_4^+$ concentration reached zero, it was returned to 200 µM within twenty-four hours. This seemed sufficient to prevent supply regime
effects on total biomass, even though the draw-down treatment only supplied about half as much nitrogen as the steady state treatment. Nonetheless, significant differences in the R:S ratio between treatments indicate that the draw-down treatment may have imposed some stress on the seedlings. Similar to the NO$_3^-$ treatment, more biomass was allocated to root production. Possibly, over a longer period, this allocation to roots would affect total biomass through a reduction in leaf area.

Family differences in both biomass and R:S ratio were evident in these two environments and there was no g x e interaction. Families 10, 3 and 1 which tended to be the largest in the N-source experiment, were the largest in this experiment. This similarity between the two experiments supports the idea that family differences in biomass are not determined by differences in uptake carriers, but rather are unrelated to N status or are determined by an inherent ability to take up more N regardless of form. The wider spread of biomass responses in the draw-down treatment also indicates that when seedlings were stressed, genetic differences in biomass accretion and N-uptake became more prominent. When seedlings received a steady supply of N, other factors responsible for differences in growth may have become more important. Family differences in R:S ratio also suggest that certain families may be more sensitive to stress because of lower uptake capacity. This stress causes generation of a higher root mass at the expense of shoot production. The negative correlation between biomass and R:S ratio in each of the treatments (data not shown) substantiates this relationship.

2. Environmental and genetic control of NUE

Efficient use of a nutrient becomes more important when that nutrient is in limited supply. Although the rate of uptake may be fairly constant as the concentration of the external solution drops from 200 μM to 0 μM, the activity of uptake carriers contributing to uptake may increase. Similar to the negative-feedback control evidenced at high concentrations to prevent NH$_4^+$ toxicity, $V_{\text{max}}$ may also respond to decreasing N concentrations. Kronzucker et al. (1996) showed induction of NH$_4^+$ uptake in the HATS concentration range by pre-treating white spruce seedlings with 100 μM NH$_4^+$. They found that the highest rates of NH$_4^+$ influx were in seedlings pretreated with NH$_4^+$ for one day after three weeks of N-starvation. Pre-treatment periods lasting longer than one day caused a decrease in the influx rate. If $V_{\text{max}}$ can increase in response to decreasing external concentrations either through synthesis of new protein carriers or activation
of pre-existing carriers, then such an effect could have occurred in the draw-down treatment. In this treatment, NUE was higher but biomass was equal to the steady-state treatment, indicating that although $V_{max}$ may have increased it was not enough to compensate for decreasing N concentrations. In addition, decreasing amounts of N caused a higher R:S ratio. When N was replenished, the higher R:S ratio and $V_{max}$ could have allowed for more N to be taken up than in the steady-state treatment causing a momentarily lower NUE which then increased as N became limiting.

The importance of genetic differences in uptake and/or allocation when N was limiting became more apparent in the draw-down treatment than in the steady-state treatment. Although families ranked similarly in both treatments, the cluster of six families in the steady-state treatment indicates that nutrients other than N might have become limiting for growth. Family shifts in NUE from high to low in the steady-state to draw-down treatments (family 9) may indicate an enhanced ability to competitively take up N at low concentrations. Conversely, a shift from low to high NUE may indicate an inability to take up low concentrations of N or simply lower uptake capacity.

3. Environmental and genetic control of WUE

In this experiment, N-stressed seedlings (draw-down treatment) developed a higher WUE both by gas exchange parameters and by C stable isotope values. This is contrary to the original expectation that seedlings provided with a low but consistent supply of N would yield higher photosynthetic capacity. Gas exchange data would hopefully have clarified the differences between $A$ and $g_s$ in the two experiments, but the only significant difference was in the $C_i/C_a$ ratio (Fig. 12). This is curious considering that $C_i/C_a$ was calculated using $A$ and $g_s$. The ratio did, however, correlate with the treatment effects on $\delta^{13}C$. Large measurement errors inherent to gas exchange measurements may account for this discrepancy.

The significant treatment effect on $\delta^{13}C$ might be explained by N-allocation. If uptake rates in fact increased during repeated draw-down cycles, and when N was plentiful a greater proportion was allocated to a smaller shoot than in the steady-state treatment, then shoot N concentrations might have been higher than in the steady-state treatment. If the N was allocated to photosynthesis, $\delta^{13}C$ would increase. This argument would be supported by equal biomasses between treatments, higher R:S ratio and C:N ratio and lower $C_i/C_a$ in the draw-down treatment.
A second possibility is that hydroponics alter the relationship between $A$ and internal N concentration through its effects on $g_s$. Elevated rates of photosynthesis and higher $g_s$ at lower $N_{\text{leaf}}$ have been reported in wheat cultivars (van den Boogaard et al., 1995). Ibrahim et al. (1998) also found that when poplar was grown in mixed high and low water and N environments, the high water, low N environment yielded the highest $g_s$, but the high water, high N environment yielded the highest $A_{\text{max}}$. Ranjith and Meinzer (1997) reported that in sugarcane grown at low levels of N fertilization, a greater proportion of N was allocated to the photosynthetic apparatus. These three papers do not by any means counter the great mass of literature that cites positive correlations between $A$ and $N_{\text{leaf}}$ (Field et al., 1986), but they do indicate that experimental conditions may influence expected treatment results.

Family differences in WUE were evident in $\delta^{13}C$ but not $C_i/C_a$. The significant treatment x family interaction indicates that unlike the N-source experiment, families changed their $\delta^{13}C$ rank between steady-state and draw-down N treatments. Under N-stress, C demand for root growth or respiration could increase $A$ and this effect could vary with family. In a study on chicory (Ameziane et al., 1995), where N-deficiency did not change root mass, shoot growth dramatically diminished in N-depleted treatments. Through $^{14}C\text{O}_2$ pulse chase experiments, they showed that this was due to an increase in the proportion of fixed carbon partitioned to the root. In a study on two peanut cultivars fertilized with high or low NH$_4$NO$_3$ (Hubick, 1990), there was no treatment effect on WUE either by stable isotope discrimination, or by the ratio of dry matter produced to water used. There was however, a g x e interaction in $A$ and root/dry matter ratio, but no effect on $g_s$. Possibly the correlation between R:S ratio and $A$ results from increasing the carbon sink strength and they are not simply two separate effects of N deprivation. There are four possible explanations for the lack of a significant difference between families in any of the gas exchange parameters: 1) the treatments were not severe enough to create measurable differences, 2) the three families selected were too similar to one another and do not represent the potential breadth of the population, 3) that $g_s$ and $A$ act in concert to maintain the $C_i/C_a$ ratio inherent to that seedling, or 4) the measurement error inherent in the technique cannot discern such fine differences in variables (Fig. 12).
B. Effects on correlated traits

1. Biomass versus $\delta^{13}$C

The negative environmental correlation between biomass and $\delta^{13}$C indicates that $\delta^{13}$C was mainly determined by $g_s$ (Virgona et al., 1996). Treatments were designed to alter $A$ more than $g_s$, but apparently this did not occur. Explanations for the correlation rely on hypotheses previously mentioned (i.e. higher shoot N concentration in draw-down treatment or unknown hydroponic effects on the $A/N_{leaf}$ correlation) (Fig. 13).

There was no genetic correlation between biomass and $\delta^{13}$C, although both traits varied significantly among families. Family differences in $A_{max}$ and stomatal responsiveness to the environment may both play a part in determining the $\delta^{13}$C of the families and the magnitude of change when placed in differing N environments. Genotypes with higher $g_s$ but limited ability to maximize $A$ with N fertilization may not change their biomass between treatments. Genotypes with greater flexibility in $A$ and more precise stomatal responses to environmental change could increase their biomass within the steady-state treatment, at the expense of WUE.

2. Biomass versus NUE

The negative environmental correlation between biomass and NUE that was seen in the N-source experiments was also observed in the N-supply experiment (Fig. 11). In both experiments, treatments that resulted in higher internal N concentrations produced greater biomass. Treatments did not yield significantly different biomasses, yet the negative correlation was significant because families clustered in a specific range in each treatment.

Within the draw-down treatment, the significant positive correlation between biomass and NUE could be due to enhanced effects of competition when N is scarce. Those seedlings that had higher uptake capacity may have removed nitrogen from the system more quickly before it became limiting. Alternatively, the advantage of highly competitive seedlings could have been not the rate of uptake, but an ability to continue uptake at very low levels of external nitrogen.

In the steady state treatment, competition for N was not a factor. Most families clustered in one area and the four that separated out presumably did so because of differing prioritization in N allocation. Certain genotypes may prioritize storage or other sinks over synthesis of photosynthetic proteins. This would alter the correlation between carbon acquisition and internal
N. Li et al. (1991) found that at low external N concentrations genetic control of NUE was equally divided between uptake, assimilation and allocation while at high external N concentrations, differences in allocation dominated genetic control. They also proposed that “families with similar levels of NUE showed large differences in the relative contribution of uptake and allocation efficiency” (Li et al., 1991).

3. NUE versus δ¹³C

A plastic correlation between NUE and δ¹³C (not evident in the N-source experiment) is clear in the N-supply rate experiment, yet it is the reverse of what would be expected (Fig. 15a). In experiments where both water and nitrogen limitations have been imposed in factorial designs, these two resource use efficiencies usually trade-off. It was expected that seedlings from the draw-down treatment would trade-off profligate water use with the efficient use of N and the opposite would be true for seedlings in the steady-state treatment. Fig. 15 shows that seedlings in the draw-down treatment had a higher NUE and δ¹³C. One possible explanation for the high NUE and less negative δ¹³C values follows the scenario used above to describe treatment effects on δ¹³C. If N concentrations in the shoot in the draw-down treatment were actually higher than in the steady-state treatment (due to induced uptake capacity at low external N concentrations), whole plant C:N ratio would be mediated by the higher R:S ratio resulting in high NUE. If the extra leaf N was allocated to photosynthesis and not structural or storage sinks, this could also yield less negative δ¹³C values.

The small number of points in the regression line limits the strength of the above argument. With only four points it is difficult to say whether the outcome was a result of chance or physiological reasons. Although the two resource use efficiencies may theoretically trade-off, hydroponics may also have interfered with this relationship in inexplicable ways.

An inherent trade-off among families was also not evident (Fig. 14b). There was a possible indication of a negative trade-off when seedlings were N stressed, but a significant correlation was not detected within this range of 10 families. In the steady-state treatment, where family differences in C:N ratio were harder to discern, there was no trade-off. Again, it is possible that the range of families was too narrow to see a trade-off, or a family trade-off was not physiologically necessary when both resources were in plentiful supply.
C. Effects on Δδ^{15}N

1. Environmental and genetic effects on Δδ^{15}N

The supply regime treatments may not have resulted in significant differences in δ^{15}N because in both treatments, inherent enzymatic discrimination was masked by an increasing concentration of ^{15}N in the media. The ^{15}N diffusion assay revealed that the fractional abundance of ^{15}N in the solution increased as N content decreased in the draw-down treatment. The significant linear regression between D and NH$_4^+$ concentration indicated that discrimination changed as the NH$_4^+$ concentration decreased (Fig. 18). Presumably, at very low concentrations the effluxed ^{15}N would be taken up again and the final plant δ^{15}N would equal that of the salt supplied. In the steady-state treatment a similar isotopic concentration gradient could have occurred, whereby δ^{15}N of the solution should have initially increased and then stabilized when the isotopic concentration gradient overcame net discrimination (Hoch et al., 1992). Because treatment solutions were refreshed only weekly to return the ^{15}N/^{14}N ratio to its original balance, the relative increase in the concentration of ^{15}N as a result of efflux forced proportionally greater uptake of ^{15}N. Ultimately, the plants in the steady-state treatment should have ended up only marginally lighter than those in the draw-down treatment.

Family differences in Δδ^{15}N in these treatments were more informative about the physiological controls of uptake, assimilation and N-demand. The intent of the experiment was to test these three aspects of NUE by treating seedlings in a draw-down system and a steady-state system. The significant family effect indicates that there was genotypic control over net discrimination. The almost significant family x treatment interaction (P<0.1) indicates that the effect of sink strength on uptake and assimilation varies for some families when placed in a repeatedly exhausted as opposed to consistently fertilized system. In order to understand where the interaction occurs, it is useful to look at family correlations between traits.

2. Correlations with biomass, NUE and δ^{13}C

Environmental correlations between Δδ^{15}N and biomass or NUE were non-significant and relatively uninformative (Fig. 14 and 16). The same two correlations on a family basis, however, may have yielded evidence of genotypic differences in uptake and utilization capacity. In the steady-state treatment, there was a positive correlation between biomass and Δδ^{15}N (Fig 15). In this treatment, families that had higher growth-driven N demand may have had higher
rates of N assimilation and less efflux (Fig. 19). Biomass and NUE were positively correlated, supporting this idea, but when NUE and δ^{15}N were plotted against one another there was not a correlation (Fig. 16).

If N uptake capacity became more important in the draw-down treatment, a negative correlation between biomass and Δδ^{15}N would occur (Fig. 14). Under these circumstance, NUE should correlate positively with Δδ^{15}N and negatively with biomass. It is possible that during draw-down cycles families that had a high uptake capacity were able to obtain a greater proportion of their N early in the cycle. If these families also had high assimilation capacity, this might have created bigger, more nitrogen use efficient seedlings (Figs. 14 and 16). Families that did not have a high uptake capacity, would have been left to assimilate lower quantities of relatively heavier N and, as a result, would be smaller. Waser et al. (1999) used a similar system to test the effect of N-starvation on the δ^{15}N of diatoms. After N starvation, when N utilization became preeminent, less N was effluxed out of the cells, reducing fractionation (Waser et al., 1999).

There was a strong positive environmental correlation between the C and N stable isotopes. The steady-state treatment yielded seedlings light in both isotopes, whereas the draw-down treatment yielded seedlings heavy in both isotopes (Fig. 17). If proportionally more nitrogen was allocated to a smaller leaf area for the synthesis of Rubisco in the draw-down treatment, this would increase δ^{13}C values. N isotope fractionation would decrease because of the regularly exhausted N-supply. In the steady-state treatment, where the R:S ratio is lower, N concentration of leaves may have been lower because the N demand was divided between multiple sinks. This could have caused lower δ^{13}C values while the build-up of^{15}N decreased N discrimination.

Within each treatment the family correlation between δ^{13}C and Δδ^{15}N could also be a combination of uptake and assimilation capacities and sink strength. The change of position along the regression lines between the two treatments may indicate that families respond differently in draw-down and steady-state treatments for the previously mentioned reasons.
CONCLUSIONS

The plastic negative correlation between WUE and NUE that was expected as a result of treatment-induced differences in internal N content did not occur. Explanations for this fall into two categories, statistical and physiological. Statistical power in an analysis of variance is dependent on the sample size, which was substantially reduced from the original experimental design. The original intent was to repeat the N-source experiment three times with one replicate of each treatment in each experiment, but the change in experimental design reduced the number of repetitions to two. The two repetitions, Experiments 1 and 2, could not be analyzed as a block design because of the significant effect of the fungal infection on the seedlings in one repetition. Within each experiment, there were only two replicates on which to conduct the analysis of variance. Such a low sample size was not sufficient to overcome the unexpected difference between replicates of the same treatment.

On a physiological basis, the low N concentrations of the media may have limited photosynthesis in all treatments despite the apparent difference in mean C:N ratio in Fig.1. Although N concentrations were different in the NO$_3^-$ and NH$_4^+$ treatments, both may have been below a threshold to cause a change in $A_{\text{max}}$. N-limitations on photosynthesis should then be supported by equal biomasses in the treatments. This did not occur indicating that a second physiological factor, potentially related to hydroponics, may have interfered with the $A_{\text{max}}$/N$_{\text{leaf}}$ relationship. This factor may have caused the significant treatment differences in $g_s$ which then affected $C_v/C_a$.

Genotypic control of all measured parameters associated with WUE, except $A$, was shown and a $g \times e$ interaction occurred only in $g_s$. Families responded similarly to each treatment indicating that either genotypic control of N-uptake carriers is linked or that WUE is unrelated to N-source. Family differentiation for NUE further supported the theory that N uptake capacity is under genetic control and maintenance of NUE rank across treatments indicates that the genetic control of differing N uptake carriers is linked. The potential dissociation between WUE and N-source could explain the lack of a trade-off between WUE and NUE seen at the family level.

$\delta^{15}$N analysis revealed further information on N relations dependent on N-source and family. On either level, environmental or genotypic, the suggested theory for N discrimination is
based on efflux/influx ratio. At the treatment level, plant $\Delta \delta^{15}N$ in the NO$_3$$^-$ treatment may have been lighter because of low storage and assimilation capacities. This caused greater efflux of heavy inorganic N. Under NH$_4^+$ fertilization, when uptake and assimilation were both high, the efflux/influx ratio was lower and a greater proportion of $^{15}N$ was assimilated. At the genotypic level, this balance between uptake and assimilation capacity could also be applied to explain family $\Delta \delta^{15}N$ values.

The third experiment, which tested N-supply rates on seedling response, produced interesting effects of the interaction between hydroponics and N-fertilization on WUE. Opposite to what was predicted, seedlings repeatedly deprived of N had a higher WUE than those given a steady supply of N. One hypothesis would be that periods of N-stress induced higher N uptake capacity. When N was re-supplied, the higher N uptake capacity supplied N to a smaller leaf area than in the steady-state treatment yielding momentary high N$_{leaf}$. The higher N$_{leaf}$ caused either higher $A$ or greater stomatal sensitivity to the environment, thereby increasing WUE and $\delta^{13}C$.

Family differences in most measured parameters were evident again in this experiment, but only in $\delta^{13}C$ was there a g x e interaction. This interaction potentially reflects family variability in water relations under these treatments. The lack of a significant family effect on gas exchange parameters was attributed to the small number of gs tested and the measurement error inherent in the technique.

Analysis of $\Delta \delta^{15}N$ values revealed that N-supply treatments had no effect on discrimination by the seedlings. In both treatments enzymatic discrimination was likely overcome by an isotope concentration gradient. The environmental correlation between C and N stable isotope ratios supports the explanation for the positive correlation between NUE and $\delta^{13}C$. Carbon isotope discrimination may have decreased in the draw-down treatment because of proportionally greater N allocation to photosynthesis during periods of high external N concentration. As the N was removed from the medium, N discrimination decreased and the seedlings became heavier. Under the steady-state treatment, proportionally less N may have been allocated to photosynthesis, possibly causing a lower N demand and less N discrimination. The build-up of $^{15}N$ in the steady-state treatment could have also decreased discrimination.

Family differences in $\Delta \delta^{15}N$ were again attributed to genotypic control of the balance between uptake and assimilation capacity. It was hypothesized that competitive ability to take
up N quickly or at low concentrations combined with a high assimilation capacity, yielded large seedlings with less negative $\Delta \delta^{15}N$ values (Table 2). When competition was not included in the treatment (steady-state), uptake ability may not have contributed as significantly to biomass, NUE and $\Delta \delta^{15}N$. The family correlation between biomass and $\Delta \delta^{15}N$ may then be a result of N demand on N assimilation capacity. Genotypic differences in photosynthetic capacity could have created high and low N demand causing the positive correlation between $\delta^{13}C$ and $\Delta \delta^{15}N$. 
RECOMMENDATIONS FOR FUTURE RESEARCH

In research experiments there are always methods which if changed might provide more information. A few of these recommendations fall in this category. Other comments are ideas for experiments to help expand the understanding of the physiology and the genetic control of N isotope discrimination. The field is relatively new with ample room for investigation. I have chosen only to propose experiments that would contribute to questions related to my research that I could not answer.

- First, I would propose that if my experiments were to be run again, a greater number of replicates both in number of experiments and number of treatment replicates should be used. This may dampen unaccounted for occurrences even in the controlled environment of a growth chamber.
- Second, if similar experiments were to be done again, I would suggest developing an on-line system for monitoring and ameliorating media N concentration. If this cannot be achieved, stabilization of N concentration could be attempted by either having low concentrations in large volume with fewer seedlings, or higher concentrations, so all NH₄⁺ is not removed within a 24 hour period.
- The use of populations from varying environments in the same type of experiment would add ecological significance to genetic control of N-uptake carriers and the relation between N-source and WUE.
- In order to further the understanding of N relations, specific studies on genetic control of N uptake kinetics would be helpful.
- Testing the organic/inorganic δ¹⁵N values of hydroponics media over time may indicate if, and in what form, heavy N is being lost from plants. Knowing if it is assimilated N or unassimilated N that is leaking back out of the plant, will clarify whether the efflux/influx ratio is controlling δ¹⁵N.
- Treatments which alter N-demand may also provide information on the contribution of efflux/influx to N discrimination. For example, phosphorus treatments, PPFD or DCMU would all affect photosynthetic demand for N without contributing to N uptake rates.
- Removal of competition for N by providing consistent renewal of ¹⁵N/¹⁴N at a constant ratio may help to test inherent genotypic discrimination.
LITERATURE CITED


## APPENDIX 1. 1/10 Modified Johnson’s solution (Johnson et al., 1957)

### Macronutrients

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<td>Fe</td>
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<td>0.112</td>
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</table>

- ml CaSO₄ are written as quantities required for Ca(NO₃)₂, (NH₄)₂SO₄, and NH₄NO₃, respectively, in order to maintain an equal final Ca concentration.

**- S concentrations differ depending on N treatment, given for Ca(NO₃)₂, (NH₄)₂SO₄, and NH₄NO₃, respectively.**
APPENDIX 2. Analysis of variance tables for all measured variables for N-source and N-supply rate experiments. Each analysis was done using a split-plot design. Pseudo-F tests were created by $\text{MS}_{\text{trmt}} = \text{MS}_{\text{TGT}} + \text{MS}_{\text{RI}} - \text{MS}_{\text{E2}}$. SS is not reported where a Pseudo-F was used because the value reported by SAS was invalid and unnecessary for creating the F-test.

Table 1. Split-plot analysis of variance for biomass (log 10 transformed) in N-source experiments. N-treatment refers to the form of nitrogen supplied.

<table>
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<tr>
<th>Source</th>
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<th>P-value</th>
<th>Error term</th>
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Table 2. Split-plot analysis of variance for R:S ratio in N-source experiments.

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<th>P-value</th>
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Table 3. Split-plot analysis of variance for $C:N \text{ ratio}$ in N-source experiments.

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Table 4. Split-plot analysis of variance for $\delta^{13}C$ in N-source experiments.

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Table 5. Split-plot analysis of variance for $\delta^{15}N$ in N-source experiments.

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Table 6. Split-plot analysis of variance for biomass in N-supply experiment. N-treatment refers to the method of NH$_4^+$ delivery (steady-state or draw-down).

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<td>Pseudo-F</td>
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Table 7. Split-plot analysis of variance for R:S ratio (log10 transformed) in N-supply experiment.

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<td>0.052</td>
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<tr>
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Table 8. Split-plot analysis of variance for C:N ratio (log10 transformed) in N-supply experiment.

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<th>F-test</th>
<th>P-value</th>
<th>Error term</th>
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<td>0.005</td>
<td>0.0003</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 9. Split-plot analysis of variance for \( \delta^{13}C \) in N-supply experiment.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>sum of squares</th>
<th>mean square</th>
<th>F-test</th>
<th>P-value</th>
<th>Error term</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-treatment</td>
<td>1</td>
<td>4.5</td>
<td>0.3099</td>
<td>11.36</td>
<td>0.05</td>
<td>Pseudo-F</td>
</tr>
<tr>
<td>Error I</td>
<td>2</td>
<td>0.368</td>
<td>0.184</td>
<td>2.86</td>
<td>0.1702</td>
<td>Error II</td>
</tr>
<tr>
<td>Family</td>
<td>9</td>
<td>7.639</td>
<td>0.849</td>
<td>13.17</td>
<td>0.0002</td>
<td>Error II</td>
</tr>
<tr>
<td>Trmt x fam</td>
<td>9</td>
<td>1.712</td>
<td>0.1902</td>
<td>2.95</td>
<td>0.0524</td>
<td>Error II</td>
</tr>
<tr>
<td>Error II</td>
<td>17</td>
<td>1.095</td>
<td>0.0644</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 10. Split-plot analysis of variance for \( \delta^{15}N \) in N-supply experiment.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>sum of squares</th>
<th>mean square</th>
<th>F-test</th>
<th>P-value</th>
<th>Error term</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-treatment</td>
<td>1</td>
<td>3.9</td>
<td>0.959</td>
<td>5.294</td>
<td>0.20</td>
<td>Pseudo-F</td>
</tr>
<tr>
<td>Error I</td>
<td>2</td>
<td>1.264</td>
<td>0.632</td>
<td>2.86</td>
<td>0.1696</td>
<td>Error II</td>
</tr>
<tr>
<td>Family</td>
<td>9</td>
<td>16.345</td>
<td>1.816</td>
<td>8.23</td>
<td>0.0002</td>
<td>Error II</td>
</tr>
<tr>
<td>Trmt x fam</td>
<td>9</td>
<td>4.93</td>
<td>0.548</td>
<td>2.48</td>
<td>0.1016</td>
<td>Error II</td>
</tr>
<tr>
<td>Error II</td>
<td>17</td>
<td>3.75</td>
<td>0.221</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX 3. Tukey test results for family ranking across all treatments used in Exp. 1 and the N-source experiment. Variables are specified below “Family”. Transformed variables used in the ANOVA tests are again used here. Families underlined by the same line are not significantly different.

EXPERIMENT 1

<table>
<thead>
<tr>
<th>Family</th>
<th>6</th>
<th>2</th>
<th>0</th>
<th>5</th>
<th>9</th>
<th>7</th>
<th>8</th>
<th>3</th>
<th>1</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomass</td>
<td>-0.661</td>
<td>-0.559</td>
<td>-0.528</td>
<td>-0.474</td>
<td>-0.419</td>
<td>-0.391</td>
<td>-0.391</td>
<td>-0.380</td>
<td>-0.305</td>
<td>-0.301</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Family</th>
<th>3</th>
<th>7</th>
<th>5</th>
<th>10</th>
<th>1</th>
<th>6</th>
<th>0</th>
<th>8</th>
<th>9</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>R:S ratio</td>
<td>0.403</td>
<td>0.415</td>
<td>0.428</td>
<td>0.430</td>
<td>0.452</td>
<td>0.452</td>
<td>0.452</td>
<td>0.513</td>
<td>0.528</td>
<td>0.658</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Family</th>
<th>8</th>
<th>9</th>
<th>0</th>
<th>2</th>
<th>6</th>
<th>5</th>
<th>1</th>
<th>7</th>
<th>10</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>C:N ratio</td>
<td>31.16</td>
<td>32.03</td>
<td>33.05</td>
<td>33.47</td>
<td>33.56</td>
<td>33.70</td>
<td>34.90</td>
<td>35.15</td>
<td>35.69</td>
<td>35.79</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Family</th>
<th>3</th>
<th>10</th>
<th>6</th>
<th>9</th>
<th>8</th>
<th>2</th>
<th>5</th>
<th>1</th>
<th>7</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ^{13}C</td>
<td>-31.04</td>
<td>-30.7</td>
<td>-30.69</td>
<td>-30.60</td>
<td>-30.46</td>
<td>-30.4</td>
<td>-30.25</td>
<td>-30.25</td>
<td>-30.24</td>
<td>-29.51</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Family</th>
<th>2</th>
<th>6</th>
<th>3</th>
<th>10</th>
<th>8</th>
<th>9</th>
<th>5</th>
<th>1</th>
<th>7</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δδ^{15}N</td>
<td>-2.64</td>
<td>-2.63</td>
<td>-2.539</td>
<td>-2.518</td>
<td>-2.504</td>
<td>-2.503</td>
<td>-2.454</td>
<td>-2.256</td>
<td>-2.069</td>
<td>-1.659</td>
</tr>
</tbody>
</table>
N-SUPPLY EXPERIMENT

<table>
<thead>
<tr>
<th>Family</th>
<th>0</th>
<th>5</th>
<th>7</th>
<th>2</th>
<th>6</th>
<th>1</th>
<th>9</th>
<th>8</th>
<th>10</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomass</td>
<td>0.66</td>
<td>0.67</td>
<td>0.71</td>
<td>0.71</td>
<td>0.76</td>
<td>0.87</td>
<td>0.90</td>
<td>0.94</td>
<td>1.108</td>
<td>1.11</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Family</th>
<th>3</th>
<th>6</th>
<th>10</th>
<th>5</th>
<th>0</th>
<th>1</th>
<th>7</th>
<th>9</th>
<th>8</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>R:S ratio</td>
<td>-0.644</td>
<td>-0.526</td>
<td>-0.508</td>
<td>-0.481</td>
<td>-0.470</td>
<td>-0.458</td>
<td>-0.450</td>
<td>-0.439</td>
<td>-0.438</td>
<td>-0.396</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Family</th>
<th>8</th>
<th>5</th>
<th>9</th>
<th>0</th>
<th>2</th>
<th>1</th>
<th>7</th>
<th>6</th>
<th>3</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>C:N ratio</td>
<td>1.386</td>
<td>1.390</td>
<td>1.397</td>
<td>1.397</td>
<td>1.405</td>
<td>1.412</td>
<td>1.417</td>
<td>1.427</td>
<td>1.450</td>
<td>1.462</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Family</th>
<th>6</th>
<th>8</th>
<th>5</th>
<th>3</th>
<th>7</th>
<th>10</th>
<th>2</th>
<th>1</th>
<th>9</th>
<th>0</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Family</th>
<th>5</th>
<th>2</th>
<th>7</th>
<th>6</th>
<th>1</th>
<th>10</th>
<th>8</th>
<th>3</th>
<th>9</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δδ¹⁵N</td>
<td>-2.08</td>
<td>-1.55</td>
<td>-1.41</td>
<td>-1.32</td>
<td>-1.23</td>
<td>-0.66</td>
<td>-0.57</td>
<td>-0.25</td>
<td>-0.18</td>
<td>0.36</td>
</tr>
</tbody>
</table>
APPENDIX 4. Summary analysis of variance table for gas exchange measurements in the N-source experiments. Scheffé multiple comparison tests were used to distinguish differences between treatments and families. Treatments or families that were significantly different are listed, e.g. 3--1.

<table>
<thead>
<tr>
<th>Source</th>
<th>d.f.</th>
<th>F-value</th>
<th>Pr &gt; F</th>
<th>F-value</th>
<th>Pr &gt; F</th>
<th>F-value</th>
<th>Pr &gt; F</th>
<th>F-value</th>
<th>Pr &gt; F</th>
<th>C_i/C_a</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>2</td>
<td>0.42</td>
<td>0.6598</td>
<td>4.10</td>
<td>0.0255</td>
<td>3.25</td>
<td>0.0509</td>
<td>3.26</td>
<td>0.0501</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Family</td>
<td>2</td>
<td>0.96</td>
<td>0.3946</td>
<td>4.22</td>
<td>0.0231</td>
<td>5.28</td>
<td>0.0101</td>
<td>3.95</td>
<td>0.0284</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trmt x fam</td>
<td>4</td>
<td>1.20</td>
<td>0.3300</td>
<td>1.7</td>
<td>0.1730</td>
<td>3.46</td>
<td>0.0179</td>
<td>0.92</td>
<td>0.4637</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>34</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scheffe/ trmt</td>
<td>none</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NH_4NO_3--NH_4^+</td>
<td>NH_4NO_3--NH_4^+</td>
<td>NH_4NO_3--NH_4^+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scheffe/ fam</td>
<td>none</td>
<td></td>
<td></td>
<td>3--1</td>
<td>3--1,3--7</td>
<td>3--7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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APPENDIX 5. Family mean correlations between treatments for each trait. The correlation coefficient (r) describes the strength of the genetic control of a trait between treatments. The correlation coefficients in bold are significant at P<0.05, α = 2 (r > 0.44).

Table 1. N-source experiment

<table>
<thead>
<tr>
<th>Trait</th>
<th>Experiment 1</th>
<th></th>
<th>Experiment 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NH₄⁺ vs NO₃⁻</td>
<td>NH₄⁺ vs NH₄NO₃</td>
<td>NH₄⁺ vs NO₃⁻</td>
<td>NH₄⁺ vs NH₄NO₃</td>
</tr>
<tr>
<td>R:S ratio</td>
<td>0.62</td>
<td>0.68</td>
<td>0.45</td>
<td>0.08</td>
</tr>
<tr>
<td>biomass</td>
<td>0.66</td>
<td>0.44</td>
<td>0.68</td>
<td>0.17</td>
</tr>
<tr>
<td>δ¹³C</td>
<td>0.54</td>
<td>0.60</td>
<td>0.79</td>
<td>0.27</td>
</tr>
<tr>
<td>C:N ratio</td>
<td>0.32</td>
<td>0.28</td>
<td>0.76</td>
<td>-0.38</td>
</tr>
<tr>
<td>Δδ¹⁵N</td>
<td>-0.35</td>
<td>0.43</td>
<td>0.19</td>
<td>0.29</td>
</tr>
</tbody>
</table>

Table 2. N-supply rate experiment

<table>
<thead>
<tr>
<th>Trait</th>
<th>steady-state vs. draw-down</th>
</tr>
</thead>
<tbody>
<tr>
<td>R:S ratio</td>
<td>0.80</td>
</tr>
<tr>
<td>biomass</td>
<td>0.49</td>
</tr>
<tr>
<td>δ¹³C</td>
<td>0.52</td>
</tr>
<tr>
<td>C:N ratio</td>
<td>0.38</td>
</tr>
<tr>
<td>Δδ¹⁵N</td>
<td>0.53</td>
</tr>
</tbody>
</table>