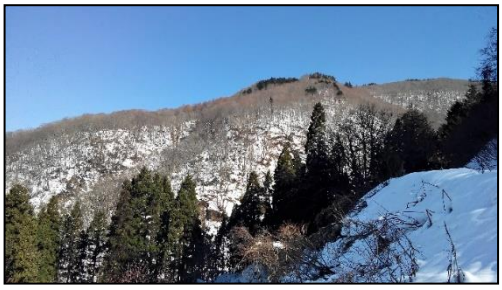
Faculty of Agriculture,
Yamagata University

UGAS
THE UNITED GRADUATE SCHOOL OF AGRICULTURAL SCIENCES, IWATE UNIVERSITY

Felix Seidel

Seasonal nutrient dynamics of four typical tree species in the mountainous regions of the Japan Sea Coast



Felix Seidel

Seasonal nutrient dynamics of four typical tree species in the mountainous regions of the Japan Sea Coast

– 日本海沿岸における山岳地域で典型的な樹種 4 種の季節的栄養動態 –

-

Department of Climate Change, Faculty of Agriculture, Yamagata University, Japan

Supervisors: Prof. M. Larry Lopez Caceres, Yamagata University, Japan

Prof. Susumu Hanayama, Yamagata University, Japan

Prof. Yoshiko Muto, Iwate University, Japan

This thesis was submitted at the Faculty of Agriculture, Yamagata University that is part of the United Graduate School of Agricultural Sciences (UGAS) to acquire the degree of *Doctor philosophiae* in the Specialty of Environmental Resource Management belonging to the Major of Biotic Environment Science.

Tsuruoka, 4th of September 2019

Content

List of relevant publications

This thesis is based on the following publications:

1. Seidel F, Lopez CML, Oikawa A, Yamanaka T. 2019. Seasonal nitrogen partitioning in Japanese cedar (*Cryptomeria japonica*, D. Don) tissues. Plant and Soil https://doi.org/10.1007/s11104-019-04178-8.

2. Seidel F, Lopez CML, Celi L, Bonifacio E, Oikawa A, Yamanaka T. 2019. Tree tissues N isotope fractionation during N intra mobilization in *Fagus crenata*. *Forests* **10**, 330 doi:10.3390/f10040330

3. Seidel F, Lopez CML, Celi L, Bonifacio E, Oikawa A, Yamanaka T. Nitrogen partitioning in Japanese larch (*Larix kaempferi*) and Black locust (*Robinia pseudoacacia*) at four phenological stages revealed by free amino acids. *In preparation*.

4. Seidel F, Lopez CML, Celi L, Bonifacio E, Kurokawa H. Seasonal nitrogen and phosphorous cycling in four Japanese cool-temperate forest species. *In preparation*.

5. Seidel F, Castaño C, Alday JG, Lopez CML, Bonet JA. Soil fungal community differences between four Japanese cool-temperate forest tree species**.** *In preparation*.

6. Enta A, Hayashi M, Lopez CML, Fujiyoshi L, Yamanaka T, Oikawa A, Seidel F. 2019. Nitrogen resorption and fractionation during leaf senescence in typical tree species in Japan. *Journal of forestry research* (*in press*)

Abstract

Background and Objectives: Nitrogen (N) and phosphorous (P) are driving elements in ecosystem development as they are important plant nutrients playing an essential part in the photosynthetic apparatus determining and limiting plant growth. N and P cycles are linked in the soil, where microorganisms and plants produce enzymes mineralizing organically bound nutrients. Species are competing for these resources and developed different mechanisms to uptake, store and recycle N and P to promote survival at different phenological stages. In general, before leaf senescence, N and P are reabsorbed from leaves and stored in the tree woody tissues with soil uptaken N and P during winter. After reabsorption is concluded, leaves will be abscised and litter quality will influence soil N and P composition. In spring, stored N and P are remobilized to support shoot growth. Yet, there is a need for quantifying the extent of N and P reabsorption and the contribution to whole-tree storage of each plant tissue as it is species dependent. To meet plant demand for N and P, many tree species formed a symbiosis with soil fungi that pass nutrients on to the host plants with ectomycorrhizal fungi playing a key role. However, for many tree species the fungal communities have not been identified. Analysing the effect of fungi on the host tree species and the movement of nutrients within hosts' plant tissues would provide insights into the mechanisms controlling plant nutrient cycling. The most common approach to study N cycling is through stable isotope analysis ($\delta^{15}N$). However, the actual transport of N remains a black box. By quantifying free amino acids content, we are able to open this box and identify the compounds transporting N. Unfortunately, tracking movement of P is more elaborate, as P is transported by proteins and only occurs in one stable isotopic composition in nature. Measuring P content instead of proteins would be more economical and may shed light on the P cycling of tree species. A detailed species dependent understanding of soil nutritional status, fungal community composition, nutrient uptake with the help of soil fungi, reabsorption, storage and remobilization along all phenological in major plant tissues at whole-tree level is necessary to improve our understanding of N and P cycling in adult trees in order to improve forest management practices.

Materials and Methods: We measured total and available N and P content, N isotope ratio and amino acids content in fine roots, coarse roots, sapwood, leaves and litter and analysed soil fungi community composition in four tree species typical of the cold-temperate forest zone in Japan: *Cryptomeria japonica* (n=9), *Larix kaempferi* (n=8), *Fagus crenata* (n=9) and

Robinia pseudoacacia (n=5). Plant tissues were sampled in four phenological stages (shoot growth, green leaf, pre- and post-abscission stage) and values upscaled to whole-tree level. *Results:* All tree species resorbed N and P in significant amounts from leaves. N was mainly stored in coarse roots during the pre-abscission stage and during the post-abscission stage in lower amounts in sapwood. P was stored in significant amounts in sapwood of *Robinia pseudoacacia* during the post-abscission period while all other species P was stored in insignificant amounts in coarse roots and/or sapwood. Growth of all plants was limited by N. *Cryptomeria japonica* seemed followed a nutrient conserving strategy as revealed by its N and P resorption and storage, making it more tolerant towards nutrient availability changes than the other species. Additionally to coarse roots and sapwood, N was stored in remaining leaves during the post-abscission stage, with younger leaves having a significantly higher N content than older leaves. Changes in isotopic composition of remaining winter leaves exposed that soil uptaken N was stored during winter in all plant tissues. In addition, free amino acids movement could explain internal N movement. In contrast, P seemed to be stored exclusively in roots and sapwood, as P was reabsorbed from abscised and remaining living leaves. The fungal communities of these plots where characterized by a divers fungal community with an absence of ectomycorrhizal fungi. 51 % of the fungal taxa remained unknown stressing the need for studies focusing on soil fungal identification to improve our understanding of this tree species nutrient cycling.

Larix kaempferi was the least N and P demanding tree species and highly N and P efficient. The movement of N could not be traced by $\delta^{15}N$, as no fractionation was found, while free amino acids proved to be valuable to unveil N transport compounds. This species was more efficient in recycling P than N and significant amounts of soil P were uptaken during times of P storage. This species was associated with a divers fungal community and an abundance of ectomycorrhizal fungi supporting the tree species nutrient uptake.

Fagus crenata had high N and P contents in plant tissues, which where most efficiently recycled. This species was rather N than P efficient as leaves N : P ratio to shifted from N to P limited conditions during time of leaf abscission. We found three phases of N storage revealed by $\delta^{15}N$ fractionation during leaf senescence: (1) reabsorption of leaf ^{15}N-depleted N to coarse roots, followed by (2) reabsorption of leaf ^{15}N-enriched N to sapwood and (3) soil ^{15}N-depleted N uptake stored in coarse roots. Further, changes in free amino acids

partially explained $\delta^{15}N$ fractionation in plant tissues. This tree species showed the lowest fungal species richness and diversity as well as a high reliance on ectomycorrhizal fungi suggesting a high specialization making this tree species more vulnerable to environmental changes.

Leguminous *Robinia pseudoacacia* was independent from soil N uptake and richer in N and P in all plant tissues with the lowest N and P reabsorption efficiency and proficiency displaying a nutrient exploitative strategy. Nevertheless, this species was N limited during the shoot growth stage shifting gradually towards N and P co-limitation during the green leaf and pre-abscission stage. The N : P ratio of litter demonstrated the dependence of this species on conserving P rather than N. Leaf reabsorbed P exceeded the storage capability of roots and sapwood, thus other plant tissues must act as an additional P storage. This tree species fungal community was very homogeneous with a high abundance of saprotrophs.

Conclusions: This study revealed new insights into the nutrient cycling of four tree species and proved that a combination of measurements of N, P, $\delta^{15}N$, and free amino acids on whole-tree level along four phenological stages with fungal community analysis is a valuable approach to improve our understanding of each tree species nutrient cycling. By linking the movement of free amino acids with N stable isotope measurements, we might have found a powerful tool to track intra-plant N movement and transformation more reliably in trees species that show significant variation in isotopic composition in plant tissues among the phenological stages. The most beneficial timing of fertilization to promote tree growth and thus C fixation from the atmosphere could be inferred from the data and was species and nutrient dependent. Based on these results we recommend the planting of *Cryptomeria japonica* in order to reduce atmospheric C as *Cryptomeria japonica* seemed to bind C more efficiently in forest soils than any other tree species. *Robinia pseudoacacia* showed similar C sequestration capabilities as *Cryptomeria japonica*, but as it is an invasive species and reduces biodiversity considerably, we do not recommend planting it. Additionally, *Cryptomeria japonica* seemed to be the most promising tree species to cope with changes in nutrient supply caused by anthropogenic influences and global change. The next steps are to identify the large number of unknown fungal species found in this study, especially for *Cryptomeria japonica* and further, analysing interactions between mycorrhizal fungi community structure with host plants internal nutrient status, in order to shed light on this symbiosis.

Chapter 1

General Introduction

Forests are the dominant terrestrial ecosystem and cover one third of its surface, having the strongest influence on the global climate, after oceans (FAO, 2010; Pan et al., 2013). They are highly biodiverse and complex systems where soil, plants and atmosphere are in constant exchange of nutrients creating the most productive terrestrial ecosystem (Pan et al., 2011; Gough, 2012). Forests provide diverse ecosystem goods and services that are valued highly around the world. There is a long history of managing forests to create an ecosystem, which can serve our demands more efficiently (McKingley et al, 2011; Pan et al., 2013), e.g., the provision of wood products for heating and construction or the protection from hydrogeomorphic hazards (e.g. snow avalanches, rockfall, landslides). However, these ecosystems are very sensitive to changes in nitrogen (N) supply (Lopez et al., 2010; Mizota et al., 2011) and in times of global warming, they may undergo changes due to possibly increasing atmospheric N deposition (Fang et al. 2009; Takebayashi et al., 2010; Fukushima et al., 2011). Thus, foresters try i.a., to keep forests ecosystems intact and additionally promote tree growth to increase wood production. With the arising challenges of global warming, forests were discovered to provide another valuable ecosystem service: They are highly efficient carbon (C) sinks (Pan et al., 2011; Gough, 2012) as they absorb more carbon than they do release, stored in soil and plants (called carbon sequestration). The C-stock in the world's forests is estimated to be 44% bound in the forest soil, 42% in the living biomass, 8% in deadwood and 5% in litter accounting in total for 861 Pentagrams of carbon with a net increase of 1.1 Pg C each year (Pan et al., 2011). Sustainable management practices would keep forests growing at a higher rate over a potentially longer period, thus providing net C sequestration benefits in comparison to unmanaged forests (Ruddell et al, 2007). Thus, increased tree growth is desired even more, as more carbon can be bound in tree tissues, forest floor and soil. However, increased growth is only possible if sufficient nutrients are available in the soil, either naturally or artificially supplied with fertilizers. As nutrients are naturally limited, uptake, reabsorption and efficient utilization of these are crucial for the survival of the species (Aerts & Chapin, 2000; Kolb & Evans, 2002; Ueda et al., 2011).

N and phosphorous (P) are among the most important plant nutrients and are driving elements in ecosystem development (Nordin et al, 2001; Reich & Oleksyn, 2004; Millard et

al., 2007; Covelo et al., 2008; Wyka et al., 2016). N and P play an essential part in the photosynthetic apparatus determining and limiting plant growth. N is part of all living cells and indispensable for the synthesis of proteins and enzymes, supporting plant seed and fruit production (Swan, HSD 1971; Koerselmann & Meuleman, 1996; Bika et al., 2018). Phosphorous is involved in the formation of sugars, starches and oils making the tree more resilient to stress, increasing root growth and blooming (Mengel 1991; Duan et al., 2008).

N and P cycles are linked in the soil, where microorganisms and plants produce enzymes mineralizing organically bound nutrients (Olander & Vitousek, 2000; Turner, 2008). When the nutrient supply of N or P in the soil is low, enzymes are triggered mineralizing nutrients that subsequently become plant available. However, when nutrient supply is high, thse enzymes are suppressed and mineralization of P ceases while mineralization of N may continue. These linked processes will limit the uptake of N and P by trees thus determining their plant tissue N and P content and subsequently during the leaf abscission stage, the leaf reabsorption efficiency and proficiency. Furthermore, with the leaf N to P ratio, we can accurately predict whether an ecosystem is N or P limited (Koerselmann & Meuleman, 1996; Güsewell & Gessner, 2009). As species are competing for these resources, they have developed different mechanisms to uptake, store and recycle N and P to promote their survival.

In general, before leaf senescence, N and P is reabsorbed from leaves and stored in the tree woody tissues with N and P uptaken from soil. This is of great adaptive significance, as these nutrients are directly available, making the trees less dependent on current nutrient availability (Chapin et al. 1990; Proe et al, 2000; Millard et al., 2001; Millard & Grelet, 2010; Wyka et al. 2016; Palacio 2018). After reabsorption concluded, leaves will be abscised and litter quality will in turn influence soil N and P composition (Güsewell & Gessner, 2009). In spring, stored N and P is remobilized from storage tissues to support shoot growth (Cooke & Weih,, 2005 ;Ueda et al., 2011). Yet, there is a need for quantifying the extent of N and P reabsorption and the contribution to whole-tree storage of each plant tissue as it is species dependent and unknown for even major plant species (Aerts & Chapin, 2000).

However, to meet the plant demand for N and P and allow them to store “excess” N and P, many tree species formed a symbiosis between their roots and soil fungi. These fungi called mycorrhiza accumulate N and P, which they pass on to the host plants, while receiving

carbon in return (Bolan, 1991; Couto-Vázquez & González-Pietro, 2010; Högberg et al., 2014). These fungi built a forest wide network between plants ("wood wide web") exchanging nutrients, water and other compounds (Hobbie & Högberg, 2012; Rhodes, 2017). Different tree species are associated with different types of mycorrhiza helping them e.g. to support growth in younger or sick trees by providing nutrients to the same species (Hobbie et al., 2009). However, for many tree species the mycorrhizal communities have not been identified, especially in Japan (Nara, 2006; Taniguchi et al., 2007; Ochimaru & Fukuda, 2007; An et al., 2008; Miyamoto et al., 2014).

Tracing the uptake of nutrients from the soil through mycorrhiza and the movement of nutrients within plant tissues would provide insights into the mechanisms controlling plant nutrient cycling. One way of tracing this movement is following the compounds transporting them: A major form of plant internal N transport is with free amino acids, which are moving with plant water from roots to leaves and back (Rai, 2002; Xu & Xiao, 2017). However, only few studies have focused on free amino acids content in adult trees and even fewer on temporal changes in plant tissues (Wyka et al., 2016). Further, these mobile compounds can transform e.g. by synthesis to proteins and thus, using only free amino acids to track N is inconclusive as these transformations are not detected (Näsholm & Ericsson, 1990; Warren et al., 2003; Warren & Adams, 2004; Harrison et al., 2009; Ueda e al., 2011).
These N transformations can be unveiled by stable isotope analysis (Templer et al., 2007). N has naturally two stable isotopes: ^{14}N and ^{15}N. The ratio of these indicates N quality and is called $\delta^{15}N$. With this tool, we can determine the N source of plants as well as draw conclusions about intra-plant N transformation and translocation processes, as many processes discriminate against either ^{14}N or ^{15}N (Millard & Proe 1993; Mead & Preston, 1994; Proe & Millard, 1994; Robinson 2001; Lopez et al., 2010; Mizota et al. 2011; Lopez et al., 2014). N isotopic discrimination in trees is not fully understood since species composition as well as biotic and abiotic factors influence physiological processes transforming N within a forest ecosystem (Peri et al., 2012; Wyka et al., 2016). However, by linking the movement of free amino acids with N stable isotope measurements, we might find a powerful tool to track N movement and transformation more reliably. Unfortunately, the application of stable isotope analysis is unsuitable for tracking movement of P, as there is only one stable P isotope. There is the possibility to use an instable radioactive form of P to get insights into P

cycling but this method is highly restricted and forbidden in many countries due to human health risks (Di et al., 1997).

A systematic understanding of the N and P cycling of tree species would enable us to improve forest management practices towards increased growth and C sequestration. Nevertheless, N and P cycling patterns are species depended (Templer et al., 2007) making sampling and analysis of each species time consuming and costly. Therefore, to allow assumptions about overlooked tree species, measurements of species belonging to different functional groups would be advisable: (1) evergreen trees seem to reabsorb N and P from shedding leaves rather to remaining leaves than to stem or roots. Additionally, leaf age influences storage and reabsorption capability (Chapin et al. 1990; Cherbuy et al, 2001; Silla & Escuerdo, 2003; Han et al., 2008). Then again, (2) deciduous trees have to resorb nutrients from all leaves and store them in the woody tissues as all leaves will be abscised (Kolb & Evans, 2002; Millard & Grelet, 2010). Lastly, (3) leguminous species can fix N from the atmosphere, thus N is always available and they may rely less on leaf N reabsorption making effective P reabsorption more significant (Rice et al., 2004; Lopez et al., 2014).

In conclusion, a detailed species dependent understanding of nutrient uptake with the help of fungal communities, nutrient reabsorption, storage and remobilization along different phenological stages in major plant tissues and soil is necessary to improve our understanding of N and P cycling in adult trees. This will enable us to adjust forest management techniques to improve their ecosystem services by promotion of forest ecosystem survival, tree growth and C fixation from the atmosphere in a world of climate change.

The aim of this work was therefore to investigate the differences in N and P cycling at four phenological stages of four typical tree species found in north eastern Japan representative for all three functional groups:

(1) Evergreen coniferous Japanese cedar (*Cryptomeria japonica*, D. Don) representing the most commercially important tree species in Japan (Sasse, 1998). (2) Deciduous coniferous Japanese larch (*Larix kaempferi*, Sarg.), representative of *Larix* species making up more than one-third of the Eurasian boreal forest (Choi et al., 2008) and deciduous broad-leaved Japanese beech (*Fagus crenata*, Blume) being the most common and widely distributed deciduous broad-leaved tree species in Japan (Liang et al., 1995, Watanabe et al., 2012). Finally, (3) leguminous broad-leaved black locust (*Robinia pseudoacacia*, L.) which is a

nitrogen fixing invasive species and the second most abundant deciduous tree species in the world (Malcom et al., 2008).

The objectives were:

(1) to quantify seasonal N and P partitioning and cycling on whole-tree level,

(2) to trace the source and sink of N intra-plant mobilization by means of $\delta^{15}N$ at whole-plant level,

(3) to find a relationship between free amino acids movement and plant tissues seasonal N isotopic composition and lastly,

(4) to identify the mycorrhizal communities and evaluate their effect on the selected species and soils.

Chapter 2

Seasonal nitrogen partitioning in Japanese cedar (*Cryptomeria japonica*, D. Don) tissues

Felix Seidel[1], M. Larry Lopez C.[1], Akira Oikawa[1], Toshiro Yamanaka[2]

[1]*Faculty of Agriculture, Yamagata University, Tsuruoka, Japan*

[2]*Tokyo University of Marine Science and Technology, Tokyo, Japan*

Abstract

Background and Objectives: Nitrogen withdrawal from senescing leaves in evergreen trees is assumed to be stored mainly in the remaining leaves at the end of the growing season. However, there is evidence that roots as well as stem tissues play a significant role in nitrogen and other nutrients storage. Therefore, the objective of this study is to clarify the seasonal nitrogen cycle in Japanese cedar trees in order to elucidate its N storage strategy.

Materials and Methods: N content, isotope ratio and amino acids content were measured in coarse roots, sapwood, leaves (separated by age), litter and buds along the growing season.

Results: Nitrogen content increased from the shoot growth to the post-abscission period with younger leaves storing more N than older ones. N from senescent leaves was reabsorbed to roots in October and to sapwood and the remaining leaves in November accounting for only 5% of whole-tree stored N. No temporal N isotopic fractionation was observed in plant tissues except for leaf enrichment during storage. The variation of amino acids in leaf tissues explained internal N transport.

Conclusions: Japanese cedar trees reabsorbed leaf N and soil N in the pre-abscission period and stored most N in roots (54%) and sapwood (20%) followed by leaves (18%) and branches (8%), respectively.

Keywords: Amino acids, Leaf age, Nitrogen recycling, Nitrogen storage, Stable isotopes, Whole-tree N

1 Introduction

Nitrogen (N) is a key element in every ecosystem controlling plant growth and productivity (Aerts & Chapin, 2000; Kolb & Evans, 2002). Plant demand for nutrients is generally met through the uptake by roots and remobilization from storage tissues after translocation of nutrients from senescing leaves promoting resilience and survival (Nordin et al, 2001; Millard et al., 2007; Covelo et al., 2008; Ueda et al., 2011; Wyka et al., 2016). Nitrogen recycling controls new shoot growth after winter as well as litter quality and thus indirectly soil chemical composition (e.g. Mae, 2004; Covelo et al., 2008; Han et al., 2008a; Ueda et al., 2011; Chávez-Vergara et al., 2015).

The most common method to study natural abundance of N transformation and cycling is through stable isotope analysis (Robinson 2001; Lopez et al., 2010; Mizota et al. 2011; Lopez et al., 2014; Seidel et al., 2019) in plant tissues and/or in soil to trace N movements within the soil-plant interface (e.g. Amundson et al., 2003; Hobbie & Ouimette, 2009; Takebayashi et al., 2010; Peri et al., 2012; Shilenkova et al., 2013; Högberg et al., 2014; Fujiyoshi et al., 2016). Isotopic fractionation provides information about and is influenced by the N source, physiological transformations in the plant (Millard & Proe 1993; Mead & Preston, 1994; Proe & Millard, 1994), rooting depth and mycorrhizal associations (Couto-Vázquez & González-Pietro, 2010; Hayashi et al., 2018). There are multiple physical, chemical and biological processes varying widely among species that discriminate against either ^{14}N or ^{15}N during uptake (e.g. mycorrhiza), transport and/or storage of nitrogen in plant tissues (Robinson, 2001; Kolb & Evans, 2002; Tateno et al., 2005; Craine et al., 2009; Hobbie & Ouimette, 2009; Couto-Vázquez & González-Pietro, 2010; Millard & Grelet, 2010; Szpak, 2014).

Mixing of N pools, temporal variation in N content and N source utilization complicates the interpretation of N isotope values (Couto-Vázquez & González-Pietro, 2010; Högberg et al., 2014; Lopez et al., 2014, 2018; Hayashi et al., 2018). Thus, N isotope discrimination in trees is still not fully understood since species composition as well as biotic and abiotic factors influence physiological processes transforming N within a forest ecosystem (Peri et al., 2012; Wyka et al., 2016).

In general, ecosystems are very sensitive to changes in N supply (Lopez et al., 2010; Mizota et al., 2011) and the increasing anthropogenic N deposition is an additional factor possibly leading to N saturated forest ecosystems as it has been already observed in Europe and America (Templer et al., 2007; Peri et al., 2012; Schmitz et al., 2019), as well as in Asia (Fang

et al. 2009; Takebayashi et al., 2010; Fukushima et al., 2011). Although, recent studies showed that the N input might have stabilized (Holmberg et al., 2018; Vuorenmaa, 2018) or even decreased (Shibata et al., 2015).

Based on these considerations, it is necessary to understand the efficient use of N by forest ecosystems and what will their response be when they are subjected to alterations in the N cycle (Saiya-Cork et al., 2002).

The biggest fraction of leaf N is bound in the form of photosynthetic active proteins followed by other major N pools like cell walls (Harrison et al., 2009; Ueda et al., 2011), free amino acids and other N-based compounds (Warren & Adams, 2004). The proportions of N stored in free amino acids vary depending on needle age with older leaves containing fewer amino acids (Xu & Xiao, 2016). At the end of the photosynthetic active phase, shortly before senescence, N is released via protein hydrolysis and reabsorbed to storage tissues (Näsholm & Ericsson, 1990; Warren et al., 2003). This leads to an increase in certain amino acids, which are then stored in other plant tissues (Näsholm & Ericsson, 1990) and remobilised in the next spring for protein synthesis and future growth (Pietilä et al., 1991; Nordin et al., 2001; Xu & Xiao, 2016). Thus, the intra-movement of free amino acids in trees can possibly influence $\delta^{15}N$ due to their abundance in ^{14}N or ^{15}N. It has been shown that amino acids have different $\delta^{15}N$ values that were measured in different organisms to reveal food-web structures where particularly glutamic acid and phenylalanine have been proven most useful (Chikaraishi et al., 2009, 2011). Enta et al. (2019) revealed a relationship between intra-plant N movement in the form of free amino acids and a change in nitrogen isotopic composition in leaves of several tree species, while Seidel et al. (2019) showed the same relationship in leaves, stem and roots along the growing season (Seidel et al., 2019).

There have been numerous studies on N cycling in controlled environments in particular with seedlings of deciduous species. In agreement with Millard & Grelet (2010) and Leberecht et al. (2016) it is necessary to verify laboratory findings with in situ studies of adult trees. In evergreen trees, it is commonly accepted that the N reabsorbed from senescing leaves is stored in the remaining leaves giving them a dual role as a photosynthetic and N storage organ, minimizing the function of roots and stem in nutrient storage (Proe et al, 2000; Millard et al., 2001; Millard & Grelet, 2010; Wyka et al. 2016; Palacio 2018). The fractionation of $\delta^{15}N$ of N moving from a source to a sink tissue determines their level of ^{15}N enrichment and provides information of where and when N is being stored because of its enrichment in

one of the storage tissue. Many studies focused on pine N cycling (e.g. Helmisaari 1992; Millard et al; 2001; Nordin et al., 2001; Wyka et al. 2016) showed that reabsorbed N from senescing leaves was stored in younger needles and translocated to emerging ones in the next year (Chapin et al. 1990; Han et al., 2008b). Older leaves of *Picea abies* were enriched in ^{15}N in comparison to younger leaves suggesting that there is discrimination with catabolic breakdown and reallocation of N depending on leaf age (Gebauer & Schulze, 1991; Gebauer et al., 1994; Kolb & Evans, 2002). Fractionation of $\delta^{15}N$ during reabsorption from senescing leaves did not occur in *Quercus rubra* and *Quercus alba* but reallocation in spring seemed to affect stem and roots' $\delta^{15}N$ ratio (Kolb & Evans 2002). However, Cherbuy et al. (2001) and Silla & Escuerdo (2003) found that evergreen *Quercus ilex* stored N in the older leaf cohorts as well as in the stem. Even though these studies did not take storage of N in roots into account, their findings led to the reassessment of N reabsorption strategies in other evergreen tree species.

In Japan, the most commercially important tree species is Japanese cedar (*Cryptomeria japonica* D. Don). These plantations cover an area of about 4.5 million hectares (Sasse, 1998) and a change in N availability is expected to affect their productivity (Ohashi et al., 1999). Japanese cedars root system, soil respiration and leaf N content have been studied in detail (Ohashi et al., 1999; Nakaji et al., 2001; Kobayashi & Tashiro, 2003; Konôpka et al., 2006; Fukushima et al., 2011) but only little is known about the N storage capability of leaves, stem and roots, N composition and isotopic fractionation. We hypothesised that N concentration in roots, stem and remaining leaves increases as they store reabsorbed N from abscising leaves or from root uptake and this increase is reflected in changes in $\delta^{15}N$ and amino acids in each of the plant tissues involved, especially under the premise that changes in amino acids concentration directly influence $\delta^{15}N$ values. Therefore, the objectives of this study are (1) to assess the role that roots and stem play in N storage; (2) to assess N reabsorption in different leaf age groups; (3) to trace the movement of N and its transformation during storage and reallocation.

2 Materials and Methods

2.1 Study site

The study site is located at the Yamagata University Research Forest (N38° 32', E139° 51'; 265 m a.s.l.) in north-eastern Japan along the Japanese Sea side in Yamagata Prefecture with humid climate with an annual mean temperature of 9.7°C (Fig. 1). This area is dominated by heavy precipitation (~3000 mm/a) with June being the driest month (90 mm) and December being the wettest (600 mm). Approximately 50% of the total precipitation falls as snow resulting in an average annual snow depth of about 3 m. The forest is characterized by sharp-sloped mountains with mean slope angles of 20° to 44° in our plots. The plots (100 m x 100 m) are located in three 50-year old plantations composed of exclusively Japanese cedar. The understory vegetation consists exclusively of broad-leaf bamboo (*Sasa veitchii*). The sites are between 300 m and 400 m a.s.l., distributed within a distance of approximately 3 km, and chosen by stand age, accessibility throughout the year and prior soil samplings to ensure comparability of the sites in terms of soil type and nutrient status. Soil profile and texture analysis indicated that in all three sites, the soils are brown forest soils (Cambisol) developed over granite with an average depth of 80 cm and a sandy-loamy (two sites) or silty-loamy texture (one site).

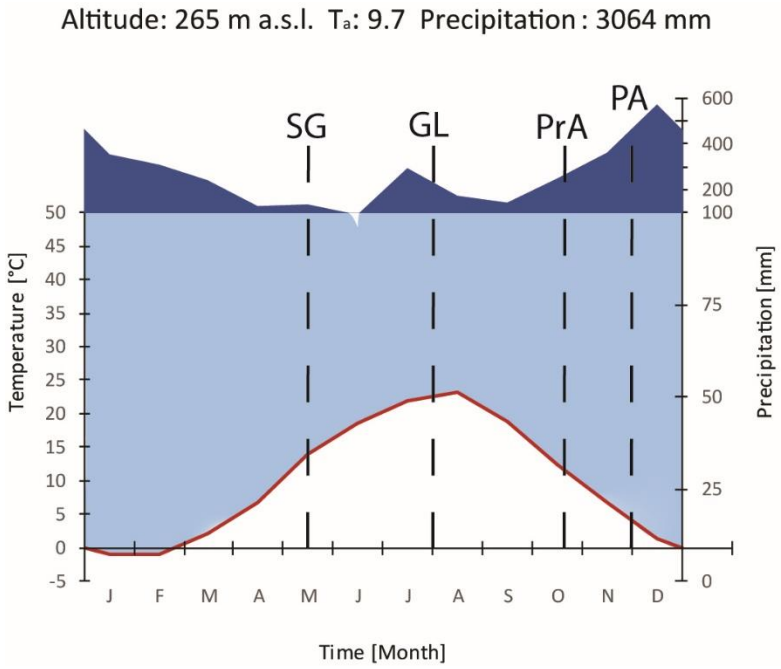


Figure 1: Meteorological conditions at the Yamagata University Research Forest. Dashed lines indicate sampling times: SG = shoot growth period, GL = green leaf period, PrA = pre-abscission period, PA = post-abscission period.

2.2 Sample collection and treatment

In 2017, three representative trees in three different slopes were sampled making a total of nine sampled trees (Table 1). Coarse roots (> 2 mm), sapwood and leaf samples were taken on May 18 (shoot growth period), August 1 (green leaf period), October 19 (pre-abscission period), and November 29 (post-abscission period). Coarse roots of single trees were dug out, identified and cut from the trunk up to a depth of 30 cm. Three samples surrounding each tree were taken and pooled. Sapwood was sampled by using an increment borer (10 mm). Assuming that there was no variation in N content, heartwood was not sampled throughout the growing season (Meerts, 2002; Tomlinson et al., 2014).

Seasonal climate variability produces phenological changes in *Cryptomeria japonica* leaves, which is formed by a set of 0 to 3 years old leaves growing in leaf cohorts, with the 0 years ones belonging to the present year. Leaf samples were taken from the lower and middle canopy from different leaf cohorts and separated by leaf age groups (0-year old, 1-year old, 2-year old and 3-year old). Leaf samples could not be obtained from the top due to restrictions in reach. During leaf senescence, leaf cohorts consisting of leaves of all age classes (0- to 3-year old leaves) change in colour from green to brown. Samples of these brown (to-be-abscised) leaf cohorts still attached to the tree, were taken on October 19, as they would eventually fall as litter later. On November 2, fresh litter samples were taken from litter traps (size: 1 m^2), which were set on May 15, 2017. Most of the litter fell in November. Finally, bud samples were taken on February 20, 2018.

Table 1: Characteristics of sampled *Cryptomeria japonica* D. Don.

Site	Coordinates	m a.s.l.	Tree	DBH	Height	Age
S 1	N38° 32.987' E139° 51.801'	295	A	41.3	21.1	54
			B	39.9	27.7	54
			C	44.6	22.5	54
S 2	N38° 33.702' E139° 50.965'	375	A	32.2	22.9	47
			B	44.5	27.3	47
			C	38.4	25.7	47
S 3	N38° 33.645' E139° 50.885'	395	A	42.3	21.4	47
			B	30.6	21.3	47
			C	34.8	17.8	47

DBH= diameter at breast height

All samples were transported in plastic bags and directly oven dried in the laboratory. Coarse roots, leaves and litter were dried at 70°C, sapwood samples at 40°C for at least 48 hrs until they were completely dry following Konôpka et al. (2006), Han et al. (2014) and Chávez-Vergara et al. (2015). Subsequently, they were ground and stored in a dark and cool place until analysis.

In August 2018, whole-tree harvest of three Japanese cedar trees was conducted to destructively collect biomass data of leaves, branches and sapwood. Before cutting the trees, branches were cut off to reduce needle loss through the falling stem. Three branches of four typical sizes (short, medium, long and very long from 120-150 cm, 150-160 cm, 200-230 cm and 300-360 cm, respectively) were selected and leaves removed and separated by age. Branches and leaves were dried at 50°C before weighing to estimate whole-tree branch and leaf (separated by years) weight. Every two meters a tree disc was cut from stems and dried at 50°C. They were separated into heart- and sapwood sections and based on the disks characteristics the volume and weight of the sapwood was calculated for all trees.

Soil samples were collected on August 24, 2017. Soil horizons were identified and characterized by colour, grain size and substrate to classify soil features (IUSS Working Group WRB, 2014) in open soil pits. For soil identification and basic soil properties, samples were taken via diagnostic horizons, while samples for nitrogen analysis were taken via increment from 0-5 cm, 5 -15 cm and 15-30 cm from three increment pits surrounding each tree. They were air-dried, sieved (< 2 mm), powdered and stored until analysis.

2.3 Analytical methods

2.3.1 Soil analysis

Soil texture was measured with a laser diffraction particle size analyzer (Coulter LS200 with an attached Fluid Module, Beckman Coulter GmbH, Germany), after treating the samples with H_2O_2 and $Na_4P_2O_7$. The analyses were triplicated. The pH was determined potentiometrically in a 1:2.5 soil:water suspension. The carbon (C) and N contents were analysed by dry combustion using the SUMIGRAPH NC-220F automatic high sensitive NC analyzer SCAS (Japan). The results were expressed on a dry-weight (105 °C) basis.

Total soluble N (TNb) was extracted from the soil samples by shaking them with 50 ml of 1M KCl. The supernatant was centrifuged, filtered (0.45 µm), stored in a refrigerator and analysed with a TOC/TNb analyser (vario TOC cube, elementar, Germany) for TNb

determination. Ammonium content was determined using the method of Crooke et al. [28], whereas nitrate content was determined by using the method of Mulvaney [29] modified by Miranda et al. [30]. The colorimetric determination of ammonium and nitrate content was conducted with a U-2000 Spectrophotometer (Hitachi, Japan).

2.3.2 Total Nitrogen and $\delta^{15}N$ Isotope analysis

For the determination of total N content and $\delta^{15}N$ in plant tissues, a Thermo Quest EA1110 Elemental Analyzer (Italy) which was connected to an IsoPrime (GV Instruments, UK) was used. The isotopic compositions of samples were expressed relative to atmospheric N_2 ($\delta^{15}N$ = 0‰) on scales normalized to the known $\delta^{15}N$ values of laboratory working standards for glycine ($\delta^{15}N$ = −0.3), which was normalized to L-glutamic acid distributed as USGS-40 ($\delta^{15}N$ = −0.2‰) by SI Science Inc., Japan. Additionally a tertiary reference material was used, namely the in-house laboratory standard acetanilide (−0.89‰). The three working standards were analysed after every eight to ten samples during CF-IRMS runs to assess the replicability of the isotope measurements and normalization. One pulse of pure N_2 reference gas from a tank reservoir ($\delta^{15}N$ = −2.5‰) was discharged into the IRMS at the beginning of each chromatogram for both standards and samples. The accuracy obtained for standards and samples during the overall analytical procedure was better than ±0.2‰.

The results of $\delta^{15}N$ were expressed as ‰ deviation, relative to atmospheric N_2 ($\delta^{15}N$= 0 ‰):

$$\delta^{15}\mathrm{N} = \left(\frac{(^{15}N/^{14}N)_{sample}}{(^{15}N/^{14}N)_{standard}} - 1 \right)$$

2.3.3 Amino acid analysis

Dried plant samples were weighed and extracted with methanol and prepared by liquid/liquid extraction and ultracentrifugation. Nineteen amino acids (Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val) were quantified by capillary electrophoresis mass spectrometry (CE-MS). The detail conditions of sample extraction, preparation, and CE-MS analysis followed Oikawa et al. (2015).

2.3.4 Whole-tree N calculations

With the data of the whole-tree sampling, total N content for sapwood, branches and leaves separated by age during the green leaf period was calculated.

For estimation of the root weight an allometric equation for Japanese cedar (Lim et al., 2013) was used:

$$W = aD^bH^C$$

Where W is the biomass of roots (g), a, b and c are constants obtained from simple regression analysis (30.248, 1.352 and 1.097, respectively), D is the diameter at breast height (cm) and H is the height (m) of the tree.

Further, estimated weights of whole stem and branches were verified with the equations used by Lim et al. (2013). This equation shows values for the green leaf period. Therefore, in order to estimate leaf weight in the shoot growth period, the fresh year (0-year) leaf weight was subtracted from the total leaf weight of the green leaf period. For the pre-abscission period, the leaf weight of the green leaf period was used because we assumed that there was no significant change in leaf biomass during these two periods. However, for the N content calculation we separated the biomass into living leaf tissues and brown to-be-abscised leaf tissues to account for the differences in N content. The weight of the brown leaves was assumed equal to litter weight measured in the post-abscission period. For the post-abscission period, the litter weight was subtracted from the green leaf weight (Table 2). For sapwood and roots, no adjustments were done since change in weight would be negligible within one year.

Litter loss of the whole tree [kg] was estimated by upscaling the values obtained from litter traps (1 m²) with the canopy projected area [m²]. Canopy projected area was calculated by measuring the canopy base in four directions for each tree.

We assumed that in the post-abscission period all N is found in the respective storage tissues (roots, sapwood, and leaves) and relocated during green leaf period to growing tissues. Thus, the whole-tree nitrogen storage was calculated as follows:

$$N_{Storage} = N_{post} - N_{green}$$

Where $N_{storage}$ is the amount of N stored in a certain tissue, N_{post} is the N content during the post-abscission period and N_{green} is the N content during green leaf period. This value was

calculated for all plant tissues as relative amounts (g N kg^{-1}) and as whole-tree absolute N storage (g N).

Table 2: Seasonal change in tissue weight [kg] of Cryptomeria japonica D. Don (n=3) with ± denoting SD.

Tissue	Shoot growth period	Green leaf period	Pre-abscission period	Post-abscission period
coarse roots	128 ± 17[c]	128 ± 17[b]	128 ± 17[c]	128 ± 17[c]
sapwood	321 ± 68[c]	321 ± 68[b]	321 ± 68[c]	321 ± 68[c]
branches	120 ± 32[c]	120 ± 32[a]	120 ± 32[c]	120 ± 32[c]
living leaves	58 ± 8[c]	102 ± 15[a]	95 ± 1[c]	95 ± 1[c]
to-be abscised leaves	-	-	6 ± 1[d]	-
litter	-	-	-	6 ± 1[a]

a = Measured with destructive whole-tree harvest.
b = Calculated with allometric equations from Lim et al. (2013).
c = Estimated. A significant change in coarse roots, sapwood and branch weight was not expected within one growing season. Shoot growth period leaf weight equaled green leaf period leaf weight minus 0-year old leaves weight. From pre- and post-abscission period leaf weight the weight of to-be-abscised leaves and litter was substracted, respectively.
d= To-be-abscised leaves weight was assumed to equal litter weight.

Nitrogen reabsorption efficiency (NRE) was calculated as:

$$NRE = \left(1 - \frac{mass\ of\ N\ in\ senesced\ leaves}{mass\ of\ N\ in\ greeen\ leaves}\right) \times 100$$

Nitrogen was expressed as nitrogen mass per leaf dry mass (mg g^{-1}). Litter, represents the senesced leaves in November, while green leaf is the matured August leaf.

As litter consists of leaves of all age groups, RE was calculated for 0-year old and 3-year old leaves. We scaled this value up to whole-tree total reabsorbed leaf N. Further, we calculated NRE* to account for leaf mass loss during N reabsorption (Factor 0.754 for conifers) following Vergutz et al. (2012), which could lead to an underestimation of 10% (van Heerwaarden et al., 2003)

Finally, nitrogen resorption proficiency (NRP) is used as a more stable indicator of plant ability to recycle nutrients than NRE. N content per leaf mass in senescing leaves was used as

NRP (Killingbeck, 1996; Yasumura et al. 2005; Yuan et al. 2005). Leaves that reduce N concentration to a lower level are more proficient in resorbing N. The NRP is expressed in % dry mass.

$$\mathrm{NRP} = mass\ of\ N\ in\ senesced\ leaves$$

2.4 Statistical analysis

One-way ANOVA was applied to determine the statistical significance of differences in nitrogen content, $\delta^{15}N$, and amino acids content. If significant differences were found, a post-hoc multiple comparison was subsequently conducted, using the Tukey-Kramer test at the significance levels of 0.05 and 0.01.

3 Results

3.1 Total nitrogen content in soil and tissues

Bulk density increased from the Ah horizon (0.5 ± 0.2 g/cm³) to the Bv horizon (0.9 ± 0.1 g/cm³), while total nitrogen, total soluble nitrogen (TNb), ammonium, nitrate and dissolved organic nitrogen (DON) decreased significantly ($P < 0.01$) from top to bottom in the soil profiles (Table 3). Throughout the whole profile, ammonium formed 19 ± 16 % of all soluble N, nitrate 4 ± 2 % and DON 76 ± 17 %.

Table 3: Soil characteristics with ± denoting SD. DON stands for dissolved organic nitrogen.

Depth [cm]	pH [-]	Total N [g N kg^{-1}]	TNb [g N kg^{-1}]	Ammonium [g N kg^{-1}]	Nitrate [g N kg^{-1}]	DON [g N kg^{-1}]	C : N	$\delta^{15}N$ (‰)
0-5	4.2 ± 0.5	5.5 ± 1.6	0.39 ± 0.09	0.14 ± 0.01	0.02 ± < 0.001	0.23 ± 0.09	11 ± 3	0.9 ± 1.3
5-15	4.4 ± 0.4	2.8 ± 1.8	0.21 ± 0.04	0.03 ± < 0.01	0.01 ± < 0.001	0.16 ± 0.06	13 ± 2	2.9 ± 1.2
15-30	4.4 ± 0.3	1.3 ± 0.4	0.13 ± 0.02	< 0.01 ± < 0.01	0.01 ± < 0.001	0.12 ± 0.01	14 ± 2	4.1 ± 1.2

From shoot growth to green leaf period coarse roots N content decreased by 50 % ($P < 0.01$) from 9.8 ± 1.6 g N kg^{-1} to 4.8 ± 1.1 g N kg^{-1} followed by an increase of 33 % ($P < 0.05$) in the pre-abscission period (6.3 ± 1.5 g N kg^{-1}) and another slight increase (ns) in the post-abscission period (7.3 ± 0.6 g N kg^{-1}) (Fig. 2b).

N content of sapwood decreased by one-third from shoot growth (1.5 ± 0.3 g N kg^{-1}) to green leaf period (1 ± 0.4 g N kg^{-1}). After the pre-abscission period (1 ± 0.4 g N kg^{-1}), a 37 % increase ($P < 0.01$) of N content (1.37 ± 0.2 g N kg^{-1}) was observed (Fig. 2a).

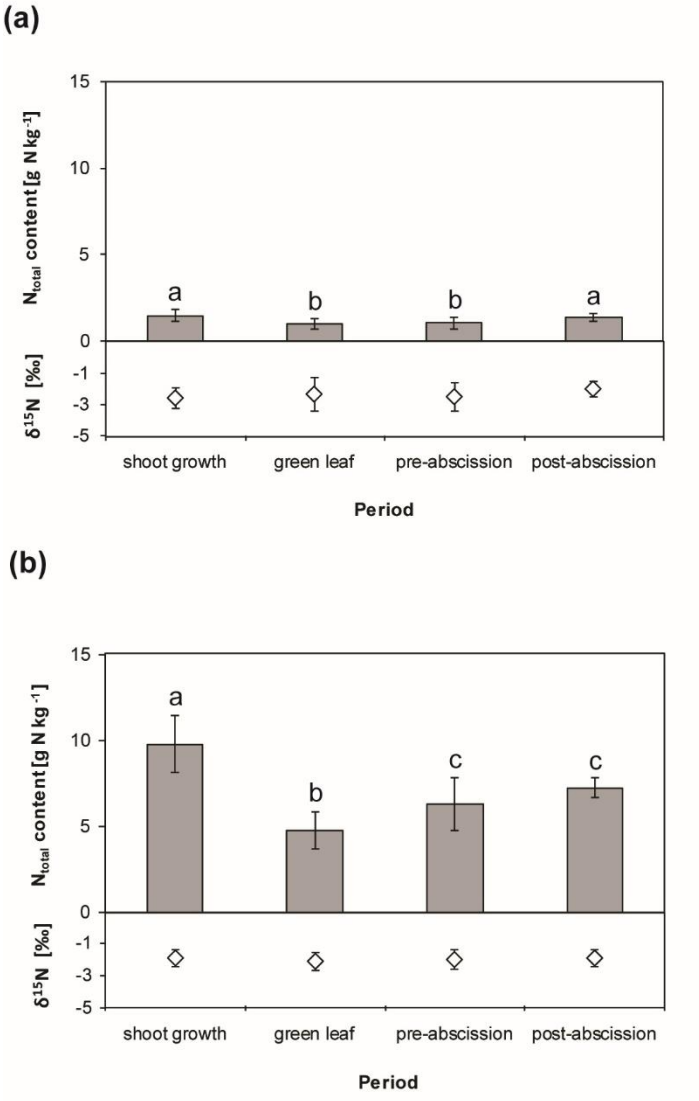


Figure 2: Seasonal pattern of N content and $\delta^{15}N$ in sapwood **(a)** and coarse roots **(b)** of *Cryptomeria japonica* D. Don. The error bars denote SD (n=9), values followed by the same letter are not significantly different (*P > 0.05*).

The overall leaf N content throughout the growing season was the highest in 0-year leaves (11.2 ± 1.7 g N kg^{-1}) and about 25 % lower ($P < 0.05$) in 3-year old leaves (8.6 ± 1.1 g N kg^{-1}) (Fig. 3a). The differences in N content throughout the growing season (all ages of leaves bulked) increased ($P < 0.01$) by 24% from the shoot growth (8.0 ± 0.6 g N kg^{-1}) to the green

leaf period (9.9 ± 1.2 g N kg^{-1}). The leaf N content remained stable through the pre-abscission period (9.8 ± 1.3 g N kg^{-1}) to finally increase again ($P < 0.01$) by 18% in the post-abscission period (11.6 ± 1.2 g N kg^{-1}) in remaining leaves. The N content in brown to-be-abscised leaf cohorts decreased ($P < 0.01$) in the pre-abscission period (5.6 ± 1.0 g N kg^{-1}) as well as in litter samples (5.7 ± 0.9 g N kg^{-1}), which were made of abscised 3-year old leaves and whole leaf cohorts in the post-abscission period. Brown to-be-abscised leaves and litter contained 32% less N ($P < 0.01$) than remaining 3-year old leaves (8.4 ± 1.1 g N kg^{-1}) and 49% less ($P < 0.01$) in comparison to remaining 0-year leaves (11.6 ± 3.1 g N kg^{-1}) in the post-abscission period. The NRE of 3-year old leaves was 34% and 49% in 0-year old leaves ($P > 0.01$) while NRE* was 51 % and 63 % for 3-year and 0-year old leaves respectively. NRP of leaves (bulk) was 0.6 %N with no difference among leave age.

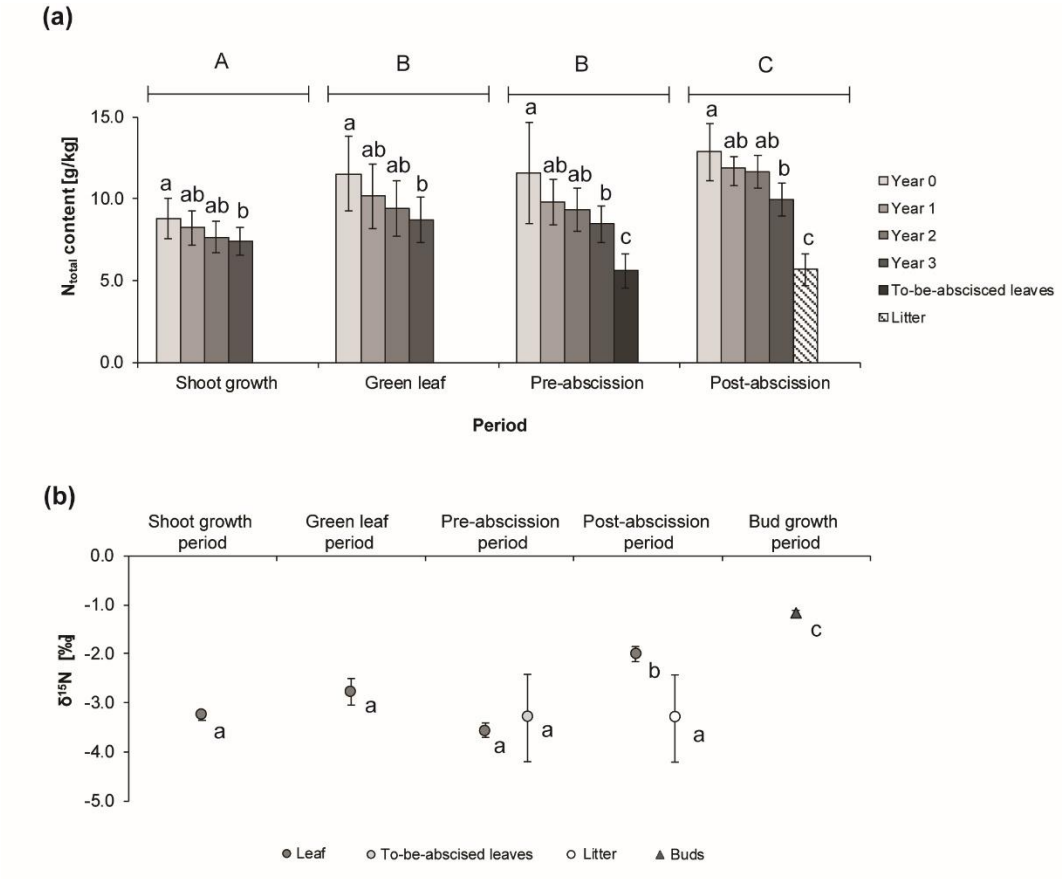


Figure 3: Seasonal pattern of N content **(a)** and $\delta^{15}N$ **(b)** in leaves divided by age from fresh leaf (Year 0) to old leaf (Year 3), litter and buds of *Cryptomeria japonica* D. Don. Capital letters show significant differences among periods while lower case letters show significant differences caused by leaf age within one period. The $\delta^{15}N$ values among leaf age did not show any significant difference thus, the depicted values are a mean of all leaf ages. The error bars denote SD (n=9), values followed by the same letter are not significantly different (*P > 0.05*).

The total increase of leaf N content from the shoot growth to the post-abscission period is 45%. Furthermore, buds produced in February (9.1 ± 2.2 g N kg^{-1}) contained similar amounts of N content compared to 0-year old leaves in the shoot growth period (8.8 ± 1.2 g N kg^{-1}) closing the N cycle of one year.

The highest relative N storage was in coarse roots (2.5 ± 1 g N kg^{-1}) ($P > 0.01$), followed by leaves (1.7 ± 0.5 g N kg^{-1}). Sapwood (0.4 ± 0.2 g N kg^{-1}) and branches (0.4 ± 0.2 g N kg^{-1}) relative N storage was low ($P > 0.01$). Among leaf age groups, the 2-year old leaves stored the highest proportion ($P > 0.01$) of leaf N (2.3 ± 0.5 g N kg^{-1}) followed by 1-year (1.8 ± 0.5 g N kg^{-1}), 0-year (1.3 ± 0.5 g N kg^{-1}) and 3-year old leaves (1.2 ± 0.5 g N kg^{-1}) (Fig. 4).

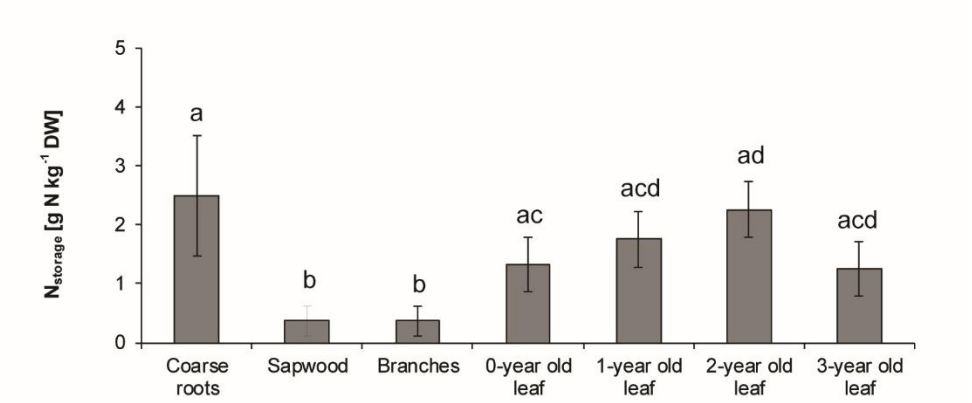


Figure 4: Relative N storage of coarse roots, sapwood, branches and leaves (separated by age group) of *Cryptomeria japonica* D. Don. The error bars denote SD (n=3), values followed by the same letter are not significantly different (*P > 0.05*).

3.2 $\delta^{15}N$ Isotope analysis

Soil $\delta^{15}N$ became four-fold heavier ($P < 0.01$) with increasing depth from 0.9 ‰ ± 1.3 (0-5 cm increment) to 4.1 ‰ ± 1.2 (15-30 cm increment). Along the season, coarse roots showed no change in $\delta^{15}N$ (-2.0 ‰ ± 0.1) (Table 3 and Fig. 1b & 2b). Similarly, sapwood $\delta^{15}N$ showed no fractionation throughout the seasons (-2.4 ‰ ± 0.3) and was similar to coarse roots (Fig. 2a). Leaf $\delta^{15}N$ among different age groups did not show any significant difference but within each age group $\delta^{15}N$ showed temporal variation; the shoot growth (-3.3 ‰ ± 0.1), the green leaf (-2.8 ‰ ± 0.3) and the pre-abscission period (-3.6 ‰ ± 0.1) as well as the litter (-3.3 ‰ ± 0.8) had similar values. However, in the post-abscission period a significantly heavier ($P < 0.01$)

$\delta^{15}N$ (-2.0 ‰ ± 0.2) was found in remaining leaves compared to other sampling periods. The buds produced in February showed the heaviest ($P < 0.01$) $\delta^{15}N$ (-1.2 ‰ ± 0.1) (Fig. 3b). Leaf $\delta^{15}N$ was significantly lighter ($P < 0.01$) than coarse roots and sapwood throughout the growing period.

3.3 Amino acid analysis in plant tissues

Free amino acids were grouped, as they together represent the mobile amino acids N pool facilitating a discussion of changes throughout the seasons. However, single amino acids in certain seasons need to be stressed in comparison to the total N pool because of its relevance on particular physiological processes as shown by (Näsholm & Ericsson, 1990). Free amino acids content in coarse roots increased two-fold ($P < 0.05$) from the shoot growth (0.19 ± 0.06 g N kg^{-1}) to the post-abscission period (0.41 ± 0.23 g N kg^{-1}) (Fig. 5a). Predominant free amino acids were alanine (Ala), asparagine (Asn), glutamic acid (Glu), glutamine (Gln), proline (Pro) and serin (Table 4). Ala, Asn, Glu and Gln contents did not change from the shoot growth to the pre-abscission period but showed a 3-fold increase ($P < 0.05$) in the post-abscission period, while no other free amino acid varied significantly over time.

The amino acids content in sapwood showed a similar pattern. There was a decrease (ns) from the shoot growth (0.06 ± 0.03 g N kg^{-1} DW) to the green leaf period (0.03 ± 0.02 g N kg^{-1} DW) followed by two fold increase ($P < 0.05$) of free amino acids in the post-abscission period (0.07 ± 0.04 g N kg^{-1}) (Fig. 5b). Predominant free amino acids were Ala, Glu, Gln and Ser. Ala increased significantly ($P < 0.05$) from the pre-abscission to the post-abscission period while Glu and Gln decreased by 40 % and 60 % ($P < 0.05$) from the shoot growth to the post-abscission period.

Total free amino acids content decreased 3-fold ($P < 0.01$) for 0-, 1-, and 2-year-old leaves from the shoot growth (0.78 ± 0.14 g N kg^{-1}) to the post-abscission period (0.27 ± 0.07 g N kg^{-1}) while 3-year old leaves showed a 2-fold decrease (0.56 ± 0.26 g N kg^{-1} and 0.26 ± 0.16 g N kg^{-1} respectively) (Fig. 5c). The most abundant free amino acids were Ala (0.13 ± 0.05 g N kg^{-1}) and Pro (0.17 ± 0.22 g N kg^{-1}). Among all measured periods in all leaves Ala contents did not vary significantly while Pro decreased by 96 % ($P < 0.01$) from the shoot growth (0.50 ± 0.19 g N kg^{-1}) to the post-abscission period (0.02 ± 0.01 g N kg^{-1}). The main decrease occurred between green leaf (0.38 ± 0.25 g N kg^{-1}) and pre-abscission period (0.03 ± 0.01 g N kg^{-1}).

Table 4: Amino acid content in different tissues of *Cryptomeria japonica* D. Don (n=3) in [g N kg^{-1}]. Only amino acids discussed in the text are displayed. SD is < 0.008.

Tissue	Period	Ala	Asn	Glu	Gln	Pro	Ser
Perennial roots	shoot growth	0.04	0.03	0.01	0.01	0.03	0.01
	green leaf	0.05	0.01	0.02	0.01	0.01	0.02
	pre-abscission	0.07	0.02	0.02	0.03	0.03	0.02
	post abscission	0.13	0.05	0.05	0.03	0.02	0.03
Sapwood	shoot growth	0.03	0.00	0.00	0.00	0.00	0.01
	green leaf	0.01	0.00	0.01	0.01	0.00	0.00
	pre-abscission	0.02	0.00	0.00	0.00	0.00	0.01
	post abscission	0.03	0.00	0.00	0.01	0.00	0.01
0-year-old leaf	shoot growth	0.11	0.01	0.01	0.00	0.64	0.01
	green leaf	0.12	0.03	0.01	0.00	0.11	0.01
	pre-abscission	0.18	0.01	0.01	0.01	0.03	0.01
	post abscission	0.09	0.00	0.01	0.00	0.02	0.01
1-year-old leaf	shoot growth	0.13	0.01	0.01	0.00	0.55	0.01
	green leaf	0.14	0.02	0.01	0.00	0.10	0.01
	pre-abscission	0.17	0.01	0.01	0.01	0.03	0.01
	post abscission	0.14	0.00	0.01	0.00	0.02	0.01
2-year-old leaf	shoot growth	0.13	0.01	0.02	0.00	0.46	0.01
	green leaf	0.13	0.02	0.01	0.00	0.10	0.01
	pre-abscission	0.14	0.01	0.01	0.01	0.03	0.00
	post abscission	0.10	0.00	0.01	0.00	0.02	0.01
3-year-old leaf	shoot growth	0.11	0.01	0.02	0.00	0.35	0.01
	green leaf	0.10	0.02	0.01	0.00	0.23	0.01
	pre-abscission	0.11	0.00	0.03	0.01	0.03	0.01
	post abscission	0.12	0.00	0.01	0.00	0.02	0.01
Litter	post abscission	0.01	0.00	0.02	0.01	0.01	0.01

Other free amino acids that were found in very low concentrations also vary over time, e.g. Glu content was low during the green leaf and high during the pre-abscission period (min. $P < 0.05$).

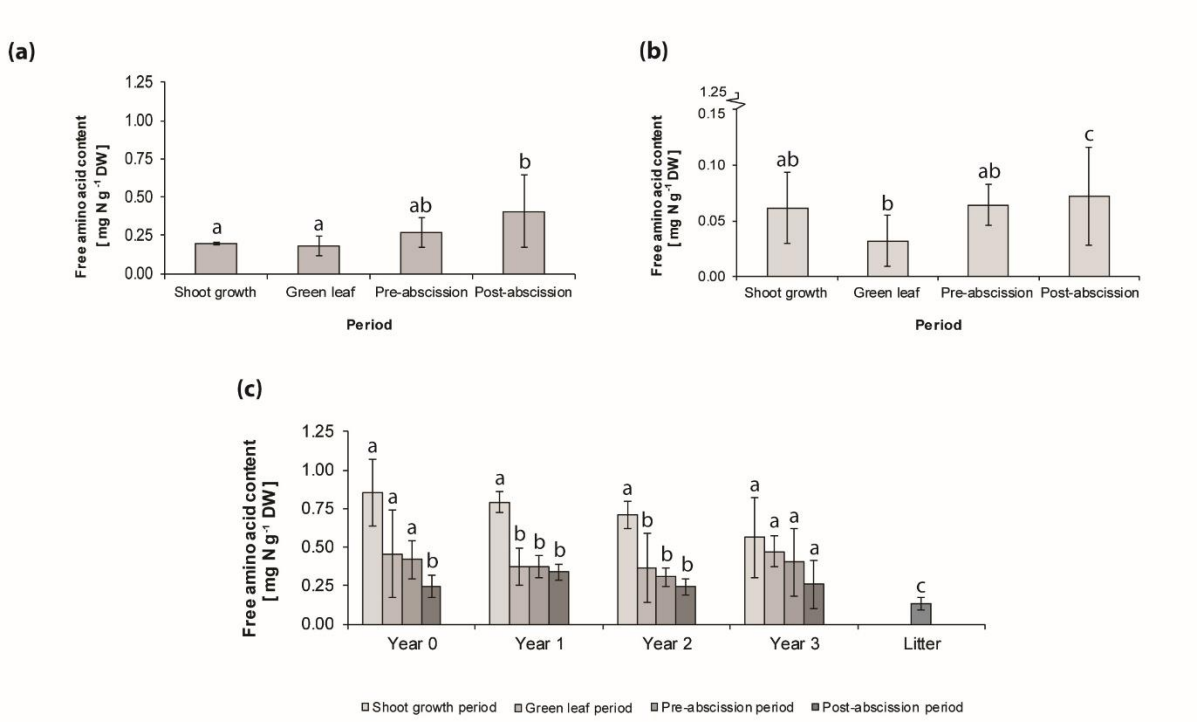


Figure 5: Seasonal pattern of free amino acids content of coarse roots **(a)** sapwood **(b)** and leaves divided by age **(c)** of *Cryptomeria japonica* D. Don. The error bars denote SD (n=3 values followed by the same letter are not significantly different (*P > 0.05*).

In the pre-abscission period only Ala, Phe and tyramine (Tyr) decreased until the post-abscission period leading to a loss of 25 % in free amino acid content with Ala accounting for 70 % of this decrease, Phe for 25 % and Tyr for 5 %.

3-year old leaves had 25 % lower concentrations of free amino acids ($P < 0.05$) in comparison to 0-year old leaves. Further, 0- and 1-year-old leaves had a higher seasonal change in abundance of 5 to 7 different free amino acids ($P < 0.01$) while in 2- and 3-year-old leaves the seasonal variation was observed for only 1 to 3 amino acids.

Total free amino acid content was low in litter ($P < 0.01$) (0.14 ± 0.03 g N kg^{-1}) in comparison to the remaining leaves.

Buds in February (0.35 ± 0.1 g N kg^{-1}) mainly contained Ala (0.10 ± 0.03 g N kg^{-1}), Pro (0.06 ± 0.03 g N kg^{-1}). In comparison to 0-year-old leaves, buds have 3 times less Pro ($P < 0.01$) while all other free amino acids had similar values as the 0-year old leaves.

Finally, in the green leaf period, leaves contained twice as much ($P < 0.05$) free amino acids (0.41 ± 0.1 g N kg^{-1}) in comparison with coarse roots (0.18 ± 0.07 g N kg^{-1}) and 14 times ($P < 0.01$) as much in sapwood (0.03 ± 0.02 g N kg^{-1}). In the pre-abscission period, leaves had 1.4 times more (ns) free amino acids (0.37 ± 0.05 g N kg^{-1}) than coarse roots (0.27 ± 0.1 g N kg^{-1}) and 6 times more ($P < 0.01$) than sapwood (0.06 ± 0.02 g N kg^{-1}). In the post-abscission period, leaves have 65 % less free amino acids (0.27 ± 0.05 g N kg^{-1}) than coarse roots (0.41 ± 0.24 g N kg^{-1}) ($P < 0.05$) and 4 times ($P < 0.05$) more than sapwood (0.07 ± 0.04 g N kg^{-1}). In the shoot growth period, leaves contained the highest amount of free amino acids (0.73 ± 0.13 g N kg^{-1}).

3.4 Whole-tree harvest

During the green leaf period, the average root, sapwood, branch and leaves weight for the three trees was 128 ± 17 kg, 321 ± 68 kg, 120 ± 32 kg, and 101 ± 15 kg, respectively. In the pre-abscission period brown to-be-abscised leaves weight was 6.3 ± 1.1 kg (Table 2).

N content of coarse roots halved from shoot growth (1254 ± 105 g N) to green leaf period (609 ± 58 g N) followed by an increase of 33 % ($P < 0.05$) in the pre-abscission period (807 ± 81 g N) and by 52 % ($P < 0.01$) in comparison to the post-abscission period (929 ± 175 g N) (Fig. 6).

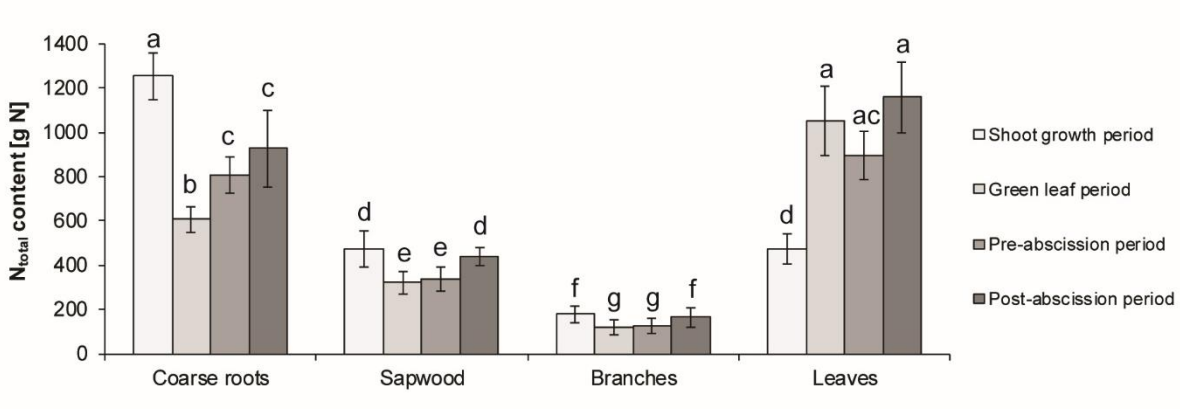


Figure 6: Seasonal pattern of total N content of coarse roots, sapwood, branches and leaves of *Cryptomeria japonica* D. Don. The error bars denote SD (n=3), values followed by the same letter are not significantly different ($P > 0.05$).

N content of sapwood decreased by 32% from shoot growth (475 ± 82 g N) to green leaf period (322 ± 70 g N) followed by an increase of 4 % (ns) to the pre-abscission period (335 ± 73 g N) and another by 31 % ($P < 0.05$) in the post-abscission period (440 ± 94 g N).

The separation of leaves by age showed that on average 43 % of all leaves are 0-year old leaves, 33 % are 1-year-old leaves, 13 % are 2-year-old leaves and 11 % are 3-year-old leaves. Taking leaf age groups and loss of N with litter into consideration, N content more than doubles ($P < 0.01$) from the shoot growth (473 ± 65 g N) to the green leaf period (1054 ± 155 g N) while it was slightly lower in the pre-abscission period (1012 ± 153 g N) followed by an increase (ns) in the post-abscission period (1158 ± 163 g N). If we do not separate the leaves by age, we would underestimate total leaf nitrogen content of whole-tree by < 5 % (ns). Whole-tree reabsorbed on average 29 ± 5 g N.

Branches N content was not analyzed, since we assumed that branches have a similar N content as the sapwood (Millard & Grelet., 2010). Thus, with data calculated from whole-tree harvest, we found a decrease ($P < 0.05$) in branches N content from the shoot growth (178 ± 37 g N) to the green leaf period (120 ± 33 g N) followed by a slight increase (ns) in N content in the pre-abscission period (125 ± 34 g N) and an increase ($P < 0.05$) in the post-abscission period of 32 % (165 ± 45 g N).

Roots stored more than twice ($P < 0.01$) as much N (320 ± 38 g N) as sapwood (118 ± 25 g N) and leaves (104 ± 21 g N), accounting for loss of N with litter. Branches stored significantly less ($P < 0.01$) N during the post-abscission period (44 ± 8 g N) (Fig. 7).

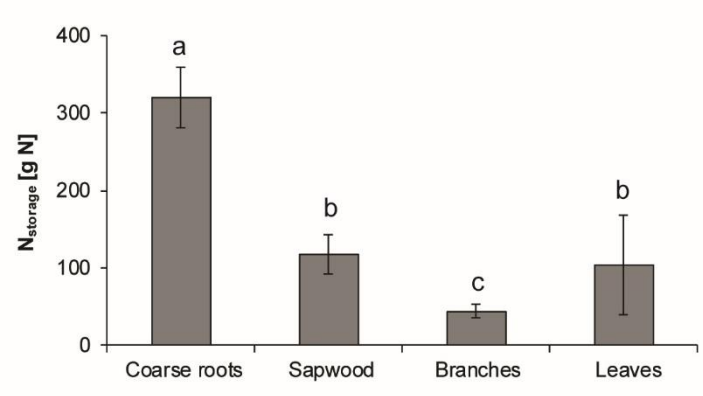


Figure 7: Total N storage of coarse roots, sapwood, branches and leaves of *Cryptomeria japonica* D. Don. The error bars denote SD (n=3), values followed by the same letter are not significantly different ($P > 0.05$).

Whole-tree stored N was 586 ± 63 g N with 54 % stored in roots, 20 % in sapwood, 18 % in leaves and 8 % in branches.

The total N content of all the measured tissues (roots, sapwood, branches and leaves) increased ($P < 0.05$) by 8 % from the green leaf period (2106 ± 33 g N) to the pre-abscission (2280 ± 45 g N) followed by an increase of 18 % ($P < 0.05$) to the post-abscission period (2691 ± 100 g N).

In the shoot growth period 20 % of N was in leaves, 20 % in sapwood, 53 % in coarse roots and 7 % in branches. In the green leaf period, 50 % of N was in leaves, 15 % in sapwood, 29 % in coarse roots and 6 % in branches. In the pre-abscission period, 43 % of N was in living leaves and 1 % in brown to-be-abscised leaves. 15 % was in sapwood, 35 % in coarse roots and 6 % in branches. In the post-abscission period, 43 % of N was found in leaves, 16 % in sapwood, 35 % in coarse roots and 6 % in branches (Fig. 6). Figure 8 shows a summary of whole tree N movement in all four periods with N content, isotopic composition and free amino acids content.

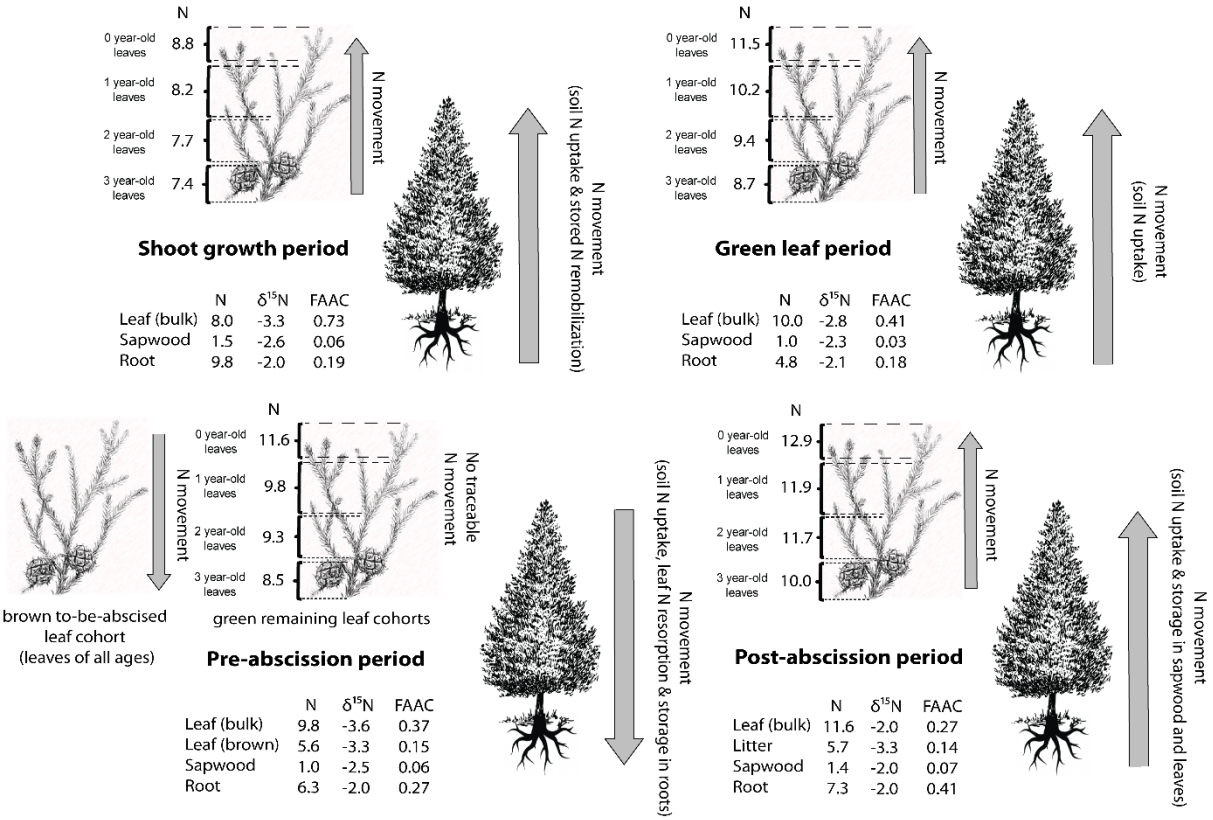


Figure 8: Summary of all measured parameters along the growing season with arrows indicating the movement of N of *Cryptomeria japonica* D. Don. N represents the amount of nitrogen [g N kg^{-1}], $\delta^{15}N$ the isotopic composition [‰] and FAAC the free amino acid content [g N kg^{-1}].

4 Discussion

4.1 Soil conditions

The decrease in soil N content and enrichment of $\delta^{15}N$ with depth are caused by input of N via precipitation and litter as well as its decomposition by microorganisms (Hobbie & Ouimette, 2009; Callesen et al., 2012, Shi et al. 2014; Zhou et al. 2014). The soil type is typical of this region and was discussed in more detail by Seidel et al. (2017).

DON represents a major soluble N pool in our soils followed by ammonium which the trees preferably uptake in comparison to nitrate (Matsui, 1995). Thus, the trees in our plots seemed to rely heavily on these sources. During uptake of soil nitrogen by coarse roots, $\delta^{15}N$ fractionation occurred due to mycorrhizal fungi selectively accumulating ^{15}N and passing on rather depleted N to the host plants (Tateno et al., 2005; Högberg et al., 2011; Hobbie & Högberg, 2012; Clemmensen et al., 2013) causing a difference of 5 ‰ between soil and root $\delta^{15}N$.

4.2 N content, reabsorption, storage & remobilization

Nitrogen content increased for all leave age groups from the shoot growth to the post-abscission period while N decreased in brown to-be-abscised leaves as well as in fresh litter. First, focussing on leaves remaining on the tree over winter, the highest N in living leaves was found in 0-year old leaves and the lowest in 3-year old leaves. This implies that older leaves relocated N to younger leaves as they aged. These results contradict previous studies (Millard & Proe 1992, 1993; Mailk & Timmer, 1997; Proe et al., 2000) showing that coniferous evergreen trees stored N in older leaves. However, Wyka et al. (2016) showed higher N concentration in Scots pine 0- and 1-year-old leaves in comparison to the 2-year-old leaves. This is in agreement with our results, concluding that leaf age may be an important factor in active N storage processes to varying degrees of storage capability. However, Millard & Grelet (2010) stated that sufficiently dense seasonal samplings have been scarce. In our study, the percentage of 3-year old leaves formed 11% of the total leaf biomass, which was comparably small. On whole-tree level, by not taking leaf age into consideration an error of > 5 % occurs, thus separation of leaf age groups becomes unnecessary for whole-tree studies but remains important for on leaf level.

When shifting the focus to the leaf cohorts that changed to a brown colour and thus will be abscised, we found that reabsorbed leaf N in the pre- and the post-abscission period led to a significant N decrease in litter. The values for NRE, NRE* and NRP found in this study are similar to values found by Enta et al. (2019) in a larch stand nearby. As the whole leaf cohort including part of the branch fell as litter, we assumed that leaf N was reabsorbed to remaining woody tissues (branch, stem, roots). This was supported by the finding of a synchrony between N stored in coarse roots and the occurrence of brown to-be-abscised leaves in the pre-abscission period implying that reabsorbed leaf N contributed to coarse roots N storage. A similar synchrony was observed between N stored in sapwood and continuous N reabsorption from senescing leaves before abscission, which was completed in November. In previous studies (Chapin et al. 1980; Malaguti et al., 2001; Millard et al., 2001; Warren et al., 2003; Millard et al., 2010; Ueda et al., 2011), N storage in roots and sapwood of deciduous trees has been described, but not the synchrony. Furthermore, studies on other evergreen trees focused on the increase of N content in the remaining leaves, after leaf senescence, because they have been considered as the main storage of N without accounting for coarse roots and sapwood (Millard et al., 2001; Millard & Grelet, 2010; Palacio 2018). Further, differences in drying temperatures might cause a difference in water content and we cannot exclude the possibility of votalization of N-containing molecules during the drying process as some samples were dried at 70°C (Derendorp, 2012). However, we considered these issues to be insignificant, as suggested by other studies that used similar drying temperatures (Moon et al, 1999; Konôpka et al. 2006; Han et al., 2014; Chávez-Vergara et al., 2015; Enta et al., 2018)

Nevertheless, our results suggest that for Japanese cedar, coarse roots and sapwood were the main storage with 74 % of whole-tree N while leaves only accounted for 18%, followed by branches (8 %). This takes into account N loss with litter fall. Only 29 g N ± 5 of whole-tree N storage (586 g N ± 63) was reabsorbed leaf N (5 %), implying that the rest of the N stored was made of soil N taken up in autumn (Titus & Kang, 1982; Tagliavini et al., 1999; Kim et al., 2009) forming 95 % of total N storage in all measured tree tissues.

The increase in N in coarse roots from post-abscission to shoot growth period suggests that uptake and storage of soil N continued throughout winter ending in remobilisation of stored N in the transition from shoot growth to green leaf period. González-Zurdo et al. (2015) found that in colder regions, the NRE and nitrogen resorption proficiency of evergreen

species leaves are lower, as more N is needed to reinforce leaf cell walls as a protection against low temperature stress. This means a greater amount of N needs to be uptaken in comparison to deciduous species resulting in a higher reliance on soil N.

4.3 Amino acids

In agreement with previous studies, the highest free amino acids content was found in leaves during the shoot growth period (Dambrine et al., 1995; Schneider et al., 1996; Weber et al., 1998) while the lowest was found in litter.

Among all plant tissues, proline was the most abundant free amino acid in leaves while alanine was most abundant in sapwood and coarse roots. Since proline is usually abundant in organisms that are under biotic or abiotic stress (Verbruggen & Hermans, 2008) the increase of proline in leaves during the shoot growth period could be related to temperature stress in our plots, which are characterized by starting their growth in April when snow depth is nearly 2 m and soil temperature is close to 0 °C (data not published). During the winter months (min. temperatures of -8.4°C) and the snowmelt period (March and April), low temperature stress (Kai & Iba, 2014) possibly triggered the biosynthesis of proline from glutamic acid in leaves in the shoot growth period. This was confirmed by the significant decrease of proline in coarse roots from post-abscission to green leaf period as it was also found by Dubey (1999). Once the temperature stress decreased, proline partly degraded to glutamic acid, which increased in the green leaf period or was synthesised with other compounds into proteins (Heuer, 1999; Verbruggen & Hermans, 2008). This glutamic acids and proline dynamic seemed to take part in N remobilization from previously stored N to fresh 0-year old leaves after bud break in the shoot growth period. Their function as important transport forms of N has been also been found for Scots pine (Näsholm & Ericsson, 1990; Verbruggen & Hermans, 2008).

Alanine is closely connected with the N metabolism (Myashita et al., 2007) by (among others) controlling ion transport and membrane permeability (Rai, 2002). This proposed function was supported by our results during the pre-and post-abscission period with peaks in alanine and N content in woody plant tissues. Further, it appeared to act as the primary form of N storage due to its stable amounts in the leaves during the whole year and its sharp increase in coarse roots and sapwood in the pre- and post-abscission period. This was in

disagreement with Näsholm & Ericsson (1990) and Nordin et al. (2001) who found that arginine was an important form of N storage in conifers. Furthermore, the increase of alanine in coarse roots and sapwood from the green leaf to the post-abscission period and its decrease in leaves from pre-abscission to post-abscission period, confirmed the reabsorption of N from leaves to coarse roots and sapwood during leaf senescence. Moreover, the increase in alanine and total free amino acids during the pre-abscission period supports the observation of the sequential storing of N first in the coarse roots in the pre-abscission and afterwards in sapwood in the post-abscission period. Thus, in the post-abscission period, Ala content is high in coarse roots and sapwood.

The significant increase of Glutamic acid and Glutamine in coarse roots in synchrony with a decrease of these amino acids in leaves and sapwood from the pre-abscission to the post-abscission period revealed the transport of N from leaves to coarse roots as it has been found for other conifers and apple trees (*Malus domestica* Borkh.) (Weber et al., 1998; Malaguti et al., 2001; Nordin et al., 2001; Guak et al., 2003).

This sharp increase in total free amino acids in the roots during the pre-abscission period could also partially be attributed to enhanced uptake of soil N from the large DON pool (Roberts & Jones, 2012) finally making up 95% of the overall stored N in trees.

There was no significant difference in free amino acids between leaf age groups throughout the growing season. N storage capacity of leaves was low in comparison to other tissues, thus, N remobilized during the shoot growth period appeared to derive mainly from coarse roots and sapwood rather than from leaves. This contradicts the findings of Weber et al. (1998) for *Picea abies* and Xu & Xiao (2016) for *Pinus massoniana* (Lamb.) who suggested that leaves were the main source of remobilized N. Further, an increase of Gln in roots and sapwood in the pre-abscission period in *Pinus sylvestris, Fagus sylvatica* and *Picea abies* was recognized as a signal of a decrease in N uptake from the soil (Muller et al, 1996; Gessler et al. 1998a; Gessler et al. 1998b; Nordin et al., 2001). However in our study, glutamine decreased, which suggests uptake of fresh soil inorganic N. This inorganic N was most likely made up of soil DON and ammonium.

4.4 $\delta^{15}N$ of all plant tissues

There was no change in coarse roots and sapwood $\delta^{15}N$ throughout the growing season as well as in leaves and litter from the shoot growth to the pre-abscission period suggesting that there was no discrimination of N during reabsorption and storage as found in earlier studies for deciduous trees (e.g. Kolb & Evans, 2002). However, in the post-abscission period, remaining leaves $\delta^{15}N$ was significantly heavier in comparison to leaves in other periods and similar to sapwood and coarse root $\delta^{15}N$. The lighter $\delta^{15}N$ signal originating from reabsorbed leaf N was dampened in roots and sapwood considering that soil N made up 95 % of N stored while reabsorbed leaf N made up only 5 %.

Further, sapwood could act as a proxy for coarse roots $\delta^{15}N$ ratio throughout the growing season and for leaves in the post-abscission period. Other studies (e.g. Elhani et al., 2003; Lopez et al., 2010) have shown that $\delta^{15}N$ is not affected by the change of sapwood to heartwood, which allows us to use tree-ring chronologies to create $\delta^{15}N$ isotope chronologies and infer long-term values of coarse roots and post-abscission period leaf $\delta^{15}N$. This constitutes valuable information of the quality of tree N absorbed temporally, which can be used to understand the impact of anthropogenic N depositions on forest ecosystems.

5 Conclusions

This study showed the timing of N and free amino acids reabsorption & storage during pre- and post-abscission period, as well as remobilization in the shoot growth period in coarse roots, sapwood and leaves. The quantification of N at whole-tree level revealed that coarse roots and sapwood were the most significant N storage tissues and not leaves as it is commonly accepted. It also showed that reabsorbed leaf N makes up only 5 % of all stored N. Additionally, whole-tree N measurements explained clearly the temporal variation of tree tissues $\delta^{15}N$. Future studies on evergreen trees N cycling need to evaluate all possible N storage tissues and not just remaining leaves and elucidate the importance of reabsorbed leaf N. Finally, leaf age separation is recommended when working at leaf level with coniferous evergreen adult trees. However, for whole-tree studies, leaf separation by age group seems irrelevant. Free amino acids content helped to explain intra-plant movement of N throughout the growing season by revealing movement of reabsorbed and remobilized leaf N to roots and sapwood and back to leaves.

Chapter 3

N Isotope Fractionation in Tree Tissues During N Reabsorption and Remobilization in *Fagus crenata* Blume

Felix Seidel[1,*], M. Larry Lopez C.[1], Luisella Celi[2], Eleonora Bonifacio[2], Akira Oikawa[1] and Toshiro Yamanaka[3]

[1]*Faculty of Agriculture, Yamagata University, Tsuruoka, Japan*

[2]*Department of Agricultural, Forest and Food Sciences, Università degli Studi di Torino, Italy*

[3]*Tokyo University of Marine Science and Technology, Tokyo, Japan*

** Correspondence: dein_Seidel@gmx.de*
Received: 19 March; Accepted: 10 April 2019; Published: date

Abstract:

Background and Objectives: Nitrogen content in tissues of *Fagus crenata* Blume is key for flowering and seed production. However, there is a lack of information on seasonal intra-plant nitrogen partitioning in this representative tree species typical of heavy snowfall regions in Japan. Therefore, the objective of this study was to elucidate *Fagus crenata* intra-plant nitrogen movement by means of nitrogen content, nitrogen isotope analysis, and amino acids temporal variability.

Materials and Methods: Nitrogen content, isotope ratio, and free amino acids content were measured in coarse roots, sapwood, leaves, and litter in four phenological stages in nine adult *Fagus crenata* trees and upscaled to the whole-tree level.

Results: Nitrogen was reabsorbed to and stored in coarse roots during the pre-abscission stage, as was revealed by the depletion of the $\delta^{15}N$ ratio of coarse roots, which coincided with an enrichment of ^{15}N found in leaves. During the post-abscission stage, N was stored in the sapwood, where an enrichment in ^{15}N was found coinciding with the depletion of the $\delta^{15}N$ ratio in leaves. It seemed that ^{15}N-enriched nitrogen was initially reabsorbed from leaves to coarse roots during the pre-abscission period, followed by the reabsorption of ^{15}N-enriched nitrogen from leaves to sapwood shortly before leaf abscission. Free amino acids content and their dynamics could mostly explain seasonal $\delta^{15}N$ fractionation in leaves, coarse roots, and partially in sapwood. At the whole-tree level, N content stored in coarse roots and sapwood was similar. Furthermore, reabsorbed leaf N accounted for 32% of all nitrogen stored during leaf senescence.

Conclusion: We found three phases of nitrogen storage revealed by $\delta^{15}N$ fractionation during leaf senescence: (1) reabsorption of leaf ^{15}N-depleted nitrogen to coarse roots, followed by (2) reabsorption of leaf ^{15}N-enriched nitrogen to sapwood and (3) soil ^{15}N-depleted nitrogen uptake to coarse roots. Further, changes in free amino acids, which are the result of enzyme activities involved in amino acids synthesis, partially explained $\delta^{15}N$ fractionation in plant tissues.

Keywords: Amino acids*;* Nitrogen recycling; Nitrogen storage; Stable isotopes; Whole-tree N

1 Introduction

Japanese beech (*Fagus crenata* Blume) is the most common and widely distributed deciduous broad-leaved tree species in Japan, growing in the cool-temperate zone as a late-successional and climax species (Liang et al., 1995, Watanabe et al., 2012). It usually grows in pure stands or mixed with *Quercus mongolica* Fisch. Ex Ledeb. and has an important ecological function, protecting soils from erosion and maintaining biodiversity. Additionally, *Fagus crenata* is used for afforestation and ceremonial plantations (Watanabe et al., 2012). The importance of these forests is reflected in the UNESCO World Natural Heritage site of the Shirakami mountains in north-eastern Japan, consisting of a natural *Fagus crenata* forest covering an area of nearly 17,000 ha. *Fagus crenata* is found in areas with deep snow, regenerating constantly and producing more seedlings and juveniles than *Fagus japonica* Maxim. (Fukushima et al., 1995). This is related to snow cover protecting the seeds from rodents and winter desiccation, as well as representing an important source of water in the shoot growth stage (Shimano & Masuzawa., 1998).

Japanese beech has developed a system of accumulating N over several years before using this storage for a controlled synchronized flowering and seed production for masting events. These events deplete the N storage of the following year, as shown in many studies (Yasumura et al., 2006; Han et al., 2008; Miyazaki et al., 2014; Han et al., 2014). This adaptation to N-limited conditions is common in most forests and the availability of mineral N rarely matches the demand of plants (Aerts & Chapin, 2000; Kolb & Evans, 2002; Yasumura et al., 2002). To allocate N for masting events, an efficient uptake of N and reabsorption of N from senescing leaves (Yasumura et al., 2005; Lopez et al., 2014) is crucial. Reabsorption takes place in the autumn during leave senescence, when N is returned to woody tissues for storage, followed by remobilization after the break of dormancy during the shoot growth stage in spring (Millard & Grelet, 2010). Remobilized leaf N greatly contributes to the growth of new tissues in spring (Han et al., 2014; Millard & Grelet, 2010). There are numerous descriptive studies on N cycling in deciduous trees, but as of now, it is still elusive how internal N sources fluctuate throughout the season in *Fagus crenata* in non-masting years (Miyazaki et al., 2014). Furthermore, there is little quantitative knowledge of seasonal allocation patterns and reabsorption efficiency (Dyckmans & Flessa, 2001). Most studies have been conducted in controlled environments with seedlings (Dyckmans & Flessa, 2001; Millard et al., 2001; Kayama et al., 2009). However,

these findings need to be validated in situ with adult trees, including the effect of soil N availability, mycorrhizae, aging of trees, and other environmental variables (Millard & Grelet, 2010; Leberecht et al. 2016). Tateno et al. (2005) that the position on the slope influenced mineralization and nitrification rates, with the bottom of the slopes showing higher nitrification and lower mineralization rates than the top, which in turn influenced ammonium and nitrate distribution along the slope. Nitrate generally has a lower $\delta^{15}N$ than ammonium, thus, a higher availability in nitrate will cause lower $\delta^{15}N$ values in mycorrhizal tissues in comparison to an abundance of ammonium in the soil (Tateno et al, 2005). Subsequently, mycorrhizal $\delta^{15}N$ will influence the host plants' $\delta^{15}N$. It has been shown that mycorrhizae allocate ^{15}N and rather pass on ^{14}N ^{14}N (Hobbie & Högberg, 2012) depleting the isotopic composition of plant tissues. This provides valuable information about the N source and internal pathways of N (Tateno et al, 2005). Soil sampling combined with seasonal samplings and N fractionation of different plant tissues could provide insights into anabolic and catabolic processes of the nitrogen metabolism of plants (Gebauer & Dietrich, 1993). Kolb & Evans (2002) hypothesized that $\delta^{15}N$ could change due to protein hydrolysis, causing fractionation within the leaf before abscission. During protein hydrolysis, soluble amino compounds are released by the degradation of proteins. These free amino acids are known to be indicators of the N status of plants (Fotelli et al., 2002). Therefore, emerging free amino acids from protein hydrolysis moving to different plant tissues may be linked to this possible isotopic fractionation. Amino acids measured in different aquatic and terrestrial organisms have shown different $\delta^{15}N$ values, and glutamic acid and phenylalanine have been proven particularly useful to reveal food-web structures (Chikaraishi et al., 2009; 2011).

The overall aim of this study is to qualitatively and quantitatively determine the temporal N partitioning of *Fagus crenata* during leaf N reabsorption in a non-masting year.

Thus, the specific objectives of this study are (1) to quantify the contribution of resorbed leaf N to the storage of the stem and roots, (2) to trace the source and sink of N intra-plant mobilization by means of $\delta^{15}N$ at the whole-plant level, and (3) to find a relation between the release of free amino acids and the seasonal N isotopic composition of plant tissues.

2 Materials and Methods

2.1 Study Site

The study site is located in north-eastern Japan in the Yamagata prefecture at the Japanese seaside. The climate is humid, with annual precipitation of 3000 mm, and approximately half corresponds to snow, which covers the sampling sites from December to May at an elevation of ~700 m above sea level. The driest month is June, with approximately 70 mm of rain and the wettest is December, with 500 mm. Nitrogen deposition with precipitation is relatively low, with 10−15 kg total N ha^{-1} $year^{-1}$ (e.g. Kanto region 30−38.5 kg total N ha^{-1} $year^{-1}$) (Watanabe et al., 2012). The mean annual temperature is 9.7 °C.

The sampling sites were distributed within a distance of 2 km on three slopes and selected by stand purity and accessibility in every season at the Research Forest of Yamagata University. To ensure comparability of the sites, soils and basic parameters were identified prior to tree sampling.

2.2 Sample Collection and Treatments

Soil samples were collected on August the 3rd in 2017 from three soil pits. Soil horizons were identified in the field following the FAO guidelines (FAO, 2006). Genetic horizons were sampled for soil characterization, while samples for nitrogen and $\delta^{15}N$ analysis were taken at fixed depths from 0−5 cm, 5 −15 cm, and 15−30 cm from three pits as replicates surrounding each individual tree. All the samples were transported in plastic bags after collection in the field, air-dried, sieved (< 2 mm), powdered, and stored until analysis. The samples used for the determination of inorganic N were frozen after transport and stored in the dark. Before analysis, they were thawed overnight in a refrigerator and sieved (< 2 mm).

Nine *Fagus crenata* trees of a similar age (70−80 years old) in the selected sites were chosen for sampling (Table 1). Coarse roots (> 2 mm), stem, and leaf samples were taken on May 20 (shoot growth stage), August 2 (green leaf stage), October 19 (pre-abscission stage), and November 29 (post-abscission stage), with fresh litter collected from litter traps (size: 1 m^2). Sapwood was sampled by using an increment borer (10 mm) and not heartwood, assuming that there was no variation in N content in heartwood throughout the growing season (Tomlinson et al., 2014). Leaf samples were taken from the lower canopy from different leaf clusters. The position in the canopy has no significant impact on the reabsorption efficiency

(Yasumura et al., 2005) and by sampling different leaf clusters, we covered possible variations in N content as leaf clusters' N content is regulated independently for this species (Osada et al., 2014).

All samples were transported in plastic bags and oven-dried in the laboratory. Coarse roots, leaves, and litter were dried at 70 °C, and sapwood samples at 40 °C, for at least 48 hrs until they were dried. Subsequently, the samples were ground and stored until analysis.

Table 1. Characteristics of sampled *Fagus crenata* Blume (*n* = 9).

Site	Coordinates	m a.s.l.	Tree	DBH	Height	Age
B 1	N38° 33.390 E139° 52.630'	670	A	30.7	25.4	71
			B	30.2	24.4	71
			C	17.0	27.5	73
B 2	N38° 33.260' E139° 52.570'	730	A	25.8	35.2	75
			B	31.4	29.1	78
			C	29.9	43.2	73
B 3	N38° 33.310' E139° 52.600'	740	A	17.0	28.1	80
			B	25.8	23.0	78
			C	26.0	31.8	76

DBH= diameter at breast height

2.3 Soil Analysis

Soil texture was measured with a laser diffraction particle size analyzer (Coulter LS200 with an attached Fluid Module, Beckman Coulter GmbH, Germany), after treating the samples with H_2O_2 and $Na_4P_2O_7$. The analyses were triplicated. The pH was determined potentiometrically in a 1:2.5 soil:water suspension. The carbon (C) and N contents were analysed by dry combustion using the SUMIGRAPH NC-220F automatic high sensitive NC analyzer SCAS (Japan). The results were expressed on a dry-weight (105 °C) basis.

Total soluble N (TNb) was extracted from the soil samples by shaking them with 50 ml of 1M KCl. The supernatant was centrifuged, filtered (0.45 µm), stored in a refrigerator and analysed with a TOC/TNb analyser (vario TOC cube, elementar, Germany) for TNb determination. Ammonium content was determined using the method of Crooke et al. (1971), whereas nitrate content was determined by using the method of Mulvaney (1996) modified by Miranda et al. (2001). The colorimetric determination of ammonium and nitrate content was conducted with a U-2000 Spectrophotometer (Hitachi, Japan).

2.3.1 Total Nitrogen and $\delta^{15}N$ Isotope Analysis

For the determination of total N content and $\delta^{15}N$ in plant tissues, a Thermo Quest EA1110 Elemental Analyzer (Italy) which was connected to an IsoPrime (GV Instruments, UK) was used. The isotopic compositions of samples were expressed relative to atmospheric N_2 ($\delta^{15}N$ = 0‰) on scales normalized to the known $\delta^{15}N$ values of laboratory working standards for glycine ($\delta^{15}N$ = −0.3), which was normalized to L-glutamic acid distributed as USGS-40 ($\delta^{15}N$ = −0.2‰) by SI Science Inc., Japan. Additionally a tertiary reference material was used, namely the in-house laboratory standard acetanilide (−0.89‰). The three working standards were analysed after every eight to ten samples during CF-IRMS runs to assess the replicability of the isotope measurements and normalization. One pulse of pure N_2 reference gas from a tank reservoir ($\delta^{15}N$ = −2.5‰) was discharged into the IRMS at the beginning of each chromatogram for both standards and samples. The accuracy obtained for standards and samples during the overall analytical procedure was better than ±0.2‰.

2.3.2 Amino Acid Analysis

Amino acids were extracted from the dried powders of plant materials with methanol and separated by liquid/liquid extraction with ultracentrifugation. Nineteen amino acids (Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val) were quantified by capillary electrophoresis mass spectrometry (CE-MS). The preparation, conditions during sample extraction, and CE-MS analysis followed Oikawa et al. (2015).

2.3.3 Whole-Tree N Calculations

Whole-tree biomass was calculated as follows:
For root, stem, and branch weight, the allometric equation by Ono et al. (2013) for *Fagus crenata* was used.

$$Y = a * DBH^b \quad (1)$$

Where, *Y* is the biomass [kg], *a* is a normalization coefficient (roots = 0.0147, stem = 0.0946, branch = 0.0049), *DBH* is the tree diameter at breast height [cm], and *b* is a scaling exponent (roots = 2.62, stem = 2.44, branch = 2.78).

Ono et al. (2013) reported that for smaller trees (DBH < 30 cm), such as those found in our study sites, leaf biomass was underestimated. Thus, for leaf biomass, the equation reported by Tateshi et al. (2010) for *Fagus sylvatica* was used.

$$\ln(ML) = p + q\ln(DBH) \quad (2)$$

Where, (*ML*) is the leaf mass [g], *p* is a normalization coefficient, *q* is a scaling exponent (2.9 and 1.7, respectively), and DBH is expressed as [cm] for *Fagus crenata*.
Fresh litter collected with litter traps verified the estimation of leaf biomass with equation (2). Lastly, for the determination of the sapwood weight, an equation by Gebauer et al. (2008) developed for *Fagus sylvatica* was used and verified by tree core sampling.

$$A_s = a * DBH^b \quad (3)$$

Where, A_s is the sapwood area (cm²), *a* and *b* are species-specific coefficients determined by regression techniques (0.778 and 1.917, respectively), and *DBH* is in [cm].
Furthermore, we assumed that in the post-abscission stage, all N was in the respective storage tissues (coarse roots and sapwood), while storage was considered empty during the green leaf stage as N is needed in the growing tissues. Thus, the whole-tree N storage was calculated as follows:

$$N_{storage} = N_{post} - N_{green} \quad (4)$$

Where, $N_{storage}$ is the amount of N stored in a certain tissue, N_{post} is the N content during the post-abscission stage, and N_{green} is the N content during green leaf stage. This value was calculated for all plant tissues as relative amounts (g N kg^{-1}) and as whole-tree absolute N storage (g N).
Reabsorption efficiency (*RE*) was calculated as

$$RE = 100 - \left(100 * \left(\frac{N_{litter}}{N_{green\ leaf}}\right)\right) \quad (5)$$

Nitrogen was expressed as nitrogen mass per leaf dry mass (mg g^{-1}). Litter represents the senesced leaves in November, while the green leaf is the matured August leaf. We used this value to calculate whole-tree total reabsorbed leaf N by considering the total tree leaf weight.

2.4 Statistical Analysis

One-way ANOVA was applied to determine the statistical significance of differences in nitrogen content, $\delta^{15}N$, and amino acids content. If significant differences were found, and a post-hoc multiple comparison was subsequently conducted, using the Tukey-Kramer test at the significance levels of 0.05 and 0.01. Furthermore, multiple linear regression analysis was conducted with RStudio (Version 1.1.463 – © 2009-2018 RStudio, Inc.) in order to evaluate the effect of the dependent variable ($\delta^{15}N$) on the independent variable (free amino acids content).

3. Results

3.1 Soil Characteristics

In all three sites, the soils were identified as brown forest soils (Cambisols), with an average depth of 80 cm and a silty-loamy texture. In the first 30 cm, we found a pH of 3.7 and a carbon to nitrogen ratio of 18 (Table 2).

Table 2: Soil characteristics with ± denoting SD. DON stands for dissolved organic nitrogen.

Depth [cm]	pH [-]	Total N [g N kg^{-1}]	TNb [g N kg^{-1}]	Ammonium [g N kg^{-1}]	Nitrate [g N kg^{-1}]	DON [g N kg^{-1}]	C : N
0-5	3.6 ± 0.1	7.8 ± 2.1	0.29 ± 0.08	0.25 ± 0.05	0.004 ± 0.006	0.04 ± 0.03	20 ± 2
5-15	3.7 ± 0.3	3.6 ± 0.9	0.17 ± 0.03	0.10 ± 0.07	0.003 ± 0.005	0.07 ± 0.07	19 ± 1
15-30	3.8 ± 0.2	1.0 ± 0.2	0.14 ± 0.05	0.10 ± 0.08	0.003 ± 0.004	0.03 ± 0.03	15 ± 1

3.2 Total Nitrogen Content in Soil and Tissues

Soil N content significantly decreased (min. $p < 0.05$) with depth from the top to deeper soil layers by 87% (Table 2, Figure 1a).

Inorganic plant available N decreased similarly ($p < 0.01$) from top to bottom by 60% and was exclusively formed of ammonium. Nitrate content accounted for less than 1% of the soil total inorganic N. Total soluble N decreased by 50% ($p < 0.01$) from top to bottom, while dissolved organic nitrogen (DON) accounted for about 25% of that with no clear distribution pattern (Table 2).

N content of coarse roots significantly decreased ($p < 0.01$) from shoot growth to the green leaf stage by 32%, followed by an increase ($p < 0.01$) of 28% during the pre-abscission stage. Finally, during the post-abscission stage, no change in N content was observed (Figure 2a).

Sapwood N content decreased by 50% ($p < 0.01$) from shoot growth to the green leaf stage, as well as in the pre-abscission stage. However, a significant increase ($p < 0.05$) in N content of 62% was observed during the post-abscission stage. However, it was 39% lower ($p < 0.01$) than the value found during the shoot growth stage (Figure 2b).

Leaves' N content significantly decreased ($p < 0.01$) from the shoot growth to the pre-abscission stage by 35%. Finally, the N content in litter was significantly ($p < 0.05$) lower in the post-abscission stage (Figure 2c), with an *RE* of 66%.

3.3 $\delta^{15}N$ Isotope Analysis

Soil $\delta^{15}N$ increased five-fold ($p < 0.01$) within the top 30 cm (Figure 1b). Coarse roots' $\delta^{15}N$ ratio depleted significantly from pre-abscission to the post-abscission stage (Figure 2a), while at the same time, sapwood $\delta^{15}N$ was significantly enriched (Figure 2b).

Leaf $\delta^{15}N$ enriched significantly during the pre-abscission stage, followed by a significant depletion in litter during the post-abscission stage (Figure 2c).

Soil $\delta^{15}N$ was the most enriched of all measured plant tissues during any given phenological stage. During the growing season, coarse roots in terms of $\delta^{15}N$ were the most depleted plant tissue, while leaves and sapwood showed similar $\delta^{15}N$ values from the shoot growth to the pre-abscission stage. Only during the post-abscission stage was leaf litter significantly more depleted in ^{15}N than sapwood.

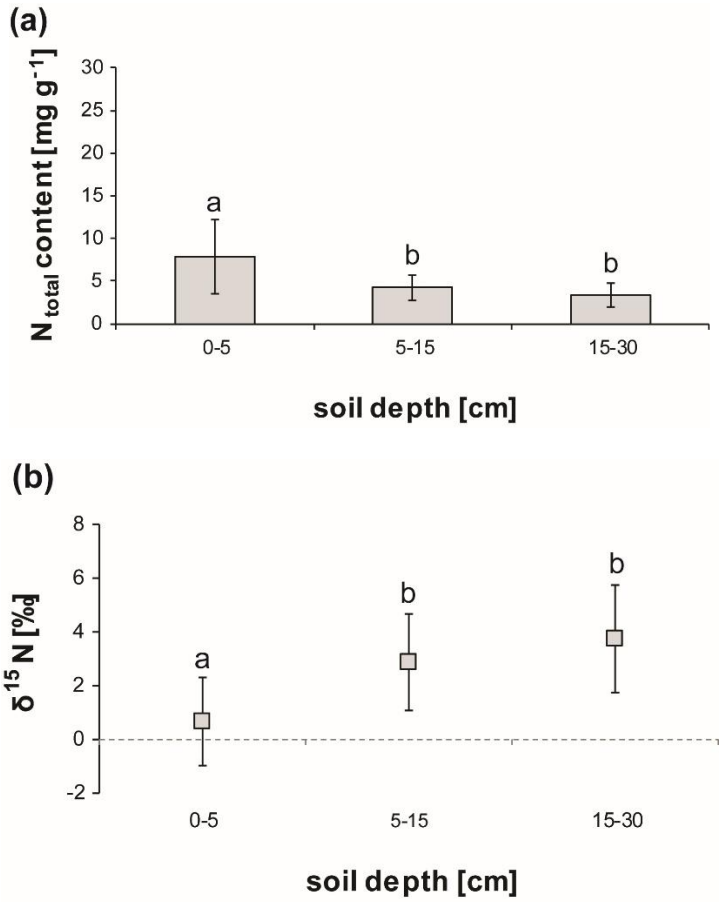


Figure 1: Total N content **(a)** and $\delta^{15}N$ **(b)** in soil layers under *Fagus crenata* Blume. The error bars denote SD (n = 9), $p < 0.05$.

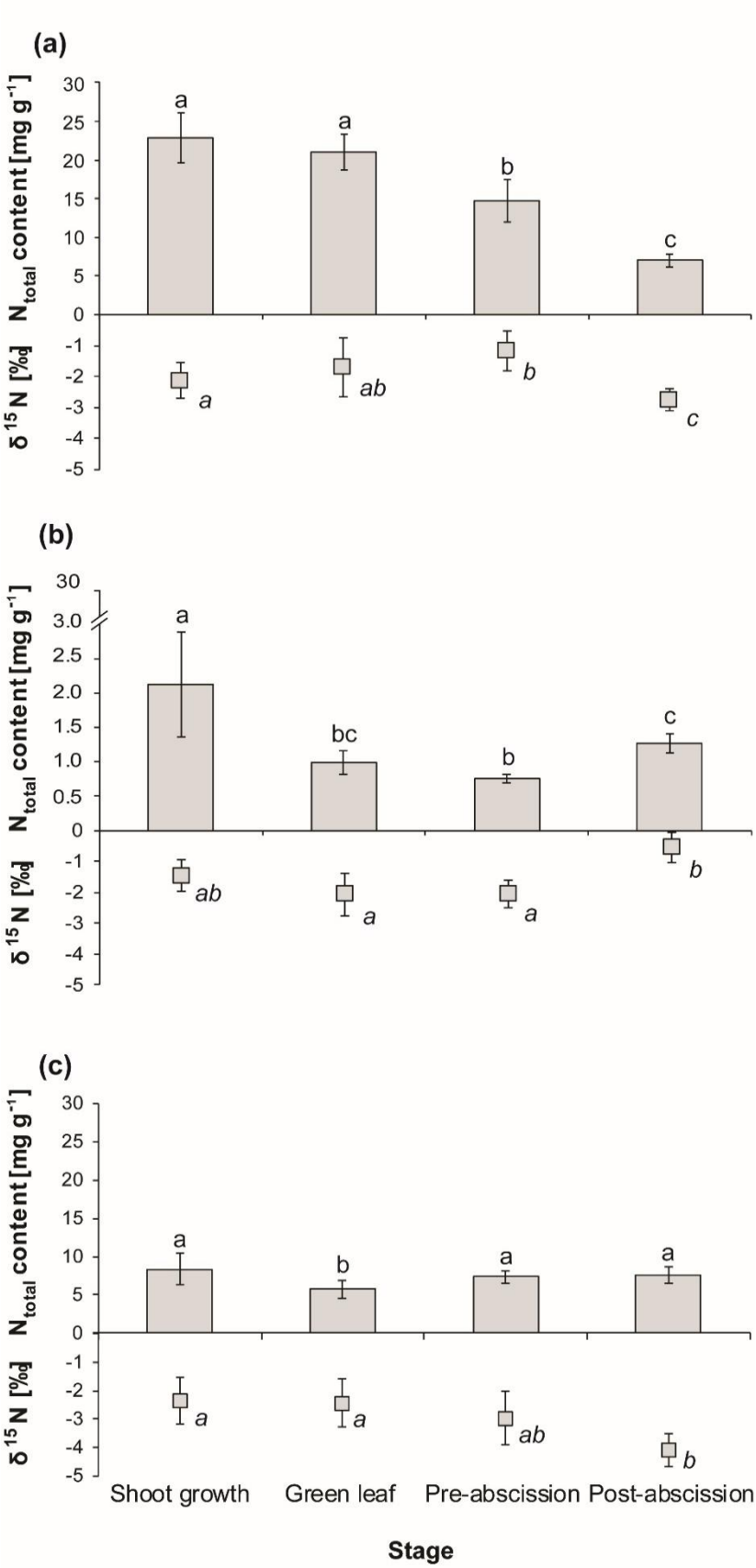


Figure 2: Seasonal pattern of N content and $\delta^{15}N$ in coarse roots **(a)**, sapwood **(b)**, and leaves **(c)** of *Fagus crenata* Blume. Leaves post-abscission stage values represent fresh litter. The error bars denote SD (n = 9), $p < 0.05$.

3.4 Amino Acid Analysis in Plant Tissues

Total free amino acids content (FAAC) in coarse roots decreased dramatically (300%, $p < 0.01$) from the shoot growth to the green leaf stage and decreased another 60% ($p < 0.05$) in the pre-abscission stage. However, it increased again during the post-abscission stage by 300% ($p < 0.05$) (Figure 3).

Sapwood FAAC only showed a significant increase ($p < 0.05$) of 50% from the green leaf stage to the post-abscission stage (Figure 3).

Leaf total FAAC decreased ($p < 0.05$) by 90% from the shoot growth to the green leaf stage. We observed a 15-fold increase ($p < 0.01$) during the pre-abscission stage and an equally sharp decrease ($p < 0.01$) in fresh litter during the post-abscission stage (Figure 3).

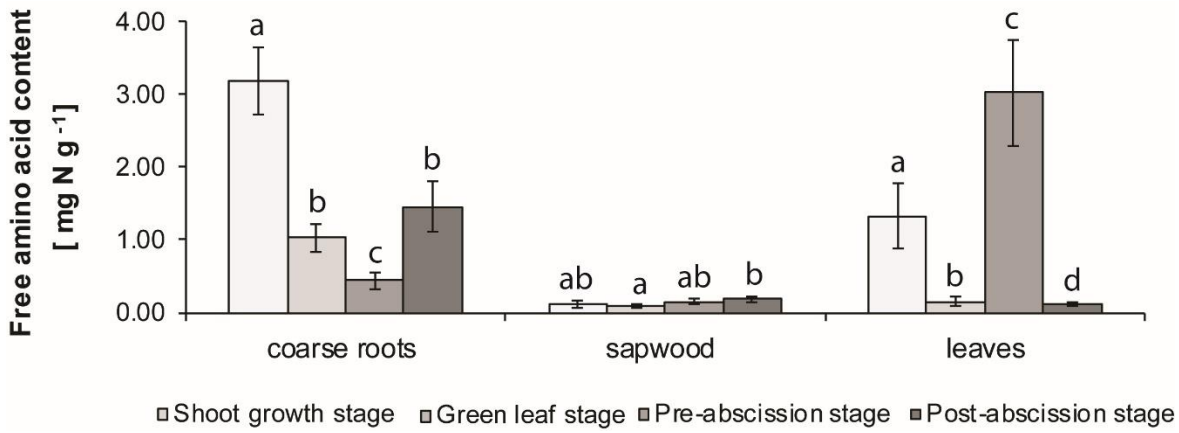


Figure 3: Seasonal pattern of free amino acids content of coarse roots, sapwood, and leaves of *Fagus crenata* Blume. Leaves post-abscission stage value represents fresh litter. The error bars denote SD ($n = 3$), $p < 0.05$.

The most abundant free amino acid in coarse roots was asparagine, which accounted for 60 to 80% of total FAAC along the growing season, with the highest content ($p < 0.05$) in the shoot growth and post-abscission stage (Table 3). Other free amino acids of minor concentration were alanine and arginine, showing a similar distribution along the growing season as asparagine. Other amino acids were found in negligible concentrations. The results of the multiple linear regression analysis showed that roots' asparagine content alone could not explain roots' $\delta^{15}N$. However, the total pool of all amino acids revealed that asparagine content has an increasing effect ($p = 0.01$) on the $\delta^{15}N$ value, while all the others have a decreasing effect ($p < 0.01$) (Table 4).

Table 3: Amino acid content in different tissues of *Fagus crenata* Blume (*n* = 3). Only amino acids found in significant amounts were reported with ± denoting SD.

Tissue	Period	Alanine [g N kg^{-1}]	Arginine [g N kg^{-1}]	Asparagine [g N kg^{-1}]
Perennial roots	shoot growth	0.34 ± 0.06	0.15 ± 0.10	2.2 ± 0.03
	green leaf	0.06 ± 0.02	0.06 ± 0.03	0.76 ± 0.09
	pre-abscission	0.05 ± < 0.01	0.03 ± 0.01	0.24 ± 0.10
	post abscission	0.18 ± 0.05	0.13 ± 0.08	0.88 ± 0.021
Sapwood	shoot growth	0.03 ± 0.10	0.00 ± 0.00	0.02 ± 0.01
	green leaf	0.02 ± 0.01	0.00 ± 0.00	0.03 ± 0.01
	pre-abscission	0.06 ± 0.01	0.00 ± 0.00	0.04 ± 0.02
	post abscission	0.06 ± 0.01	0.01 ± <0.01	0.07 ± 0.02
leaf	shoot growth	0.63 ± 0.28	0.00 ± 0.00	0.00 ± 0.00
	green leaf	0.05 ± 0.02	0.00 ± 0.00	0.00 ± 0.00
	pre-abscission	1.02 ± <0.01	0.01 ± <0.01	0.14 ± 0.01
Litter	post abscission	0.02 ± <0.01	0.01 ± <0.01	0.09 ± 0.05

Table 4: Summary statistics of the multiple regression analysis of the effect of 19 free amino acid contents on $\delta^{15}N$ in plant tissues of *Fagus Crenata* Blume (*n* = 3).

Tissue	Amino acid	Estimate	R^2	adjusted R^2	*P*
roots	Asparagine	1.52	0.67	0.60	**0.002****
	Other[1]	-2.90			**0.003****
sapwood	Alanine	-14.53	0.53	0.36	**0.026***
	Asparagine	37.18			0.349
	Other[1]	0.38			0.976
leaves	Alanine	1.56	0.76	0.73	**0.013***
	Other[1]	0.87			**0.001****

[1] sum of all other measured free amino acids

Significant values are highlighted in bold (*$P < 0.05$. **$P < 0.01$)

Asparagine was also predominant in sapwood samples, with an increasing concentration ($p < 0.01$) from shoot growth to the post-abscission stage. Along with asparagine, alanine was found in high concentrations with similar amounts and increased ($p < 0.05$) throughout the growing season. Together, they accounted for at least 50% of all free amino acids found in sapwood during any given time. Further amino acids detected in minor concentrations were glutamic acid, glutamine, aspartic acid, proline, and serine. All other amino acids were found in negligible concentrations. The results of the multiple linear regression analysis showed that sapwood asparagine content had an increasing effect ($p < 0.05$) on sapwood $\delta^{15}N$ (Table 4).

In leaves, alanine was the most abundant free amino acid, with peak concentrations ($p < 0.05$) in the shoot growth and pre-abscission stage. All other amino acids were found in negligible concentrations, except during the pre-abscission stage, where asparagine, glutamic acid, glutamine, isoleucine, leucine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine were detected. In fresh litter, during the post-abscission stage, mainly tyrosine, valine, proline, threonine, isoleucine, asparagine, leucine, phenylalanine, and tryptophan remained, while other free amino acids were depleted. The results of the multiple linear regression analysis showed that leaf alanine has the strongest control ($p < 0.05$) on leaf $\delta^{15}N$ than all the other free amino acids (Table 4).

3.5 Whole-Tree Level

The total biomass of all plant tissues was calculated for four trees of similar DBH in the green leaf stage (Table 5). We assumed that root, sapwood, and branch biomass did not change significantly during one growing season. Furthermore, leaf biomass was assumed not to change significantly during the pre-abscission stage and to equal the litter biomass in the post-abscission stage. We could not make any assumption on the leaf biomass during the shoot growth stage, thus it was not calculated for this stage (Table 5).

In the green leaf stage, the whole-tree roots' N content was 688 ± 67 g N, while sapwood, branches', and leaves' total N content was 290 ± 35 g N, 54 ± 8 g N, and 193 ± 20 g N, respectively (Table 5). In the pre-abscission stage, N content in roots increased by 25% ($p < 0.05$), while sapwood and branches showed no variation. Furthermore, we observed a 25% decrease ($p < 0.05$) of N in leaves. Finally, in the post-abscission stage, roots' N content did not change, while we found a 60% increase ($p < 0.01$) in sapwood and branches and another 55% decrease ($p < 0.01$) in litter.

Roots' N storage was 23% higher (ns) than sapwood, while N stored in branches was the lowest (Figure 4). At the whole-tree level, N stored in all measured tissues was 402 ± 93 g, of which 32% were reabsorbed from senescing leaves during leaf abscission.

Table 5: Biomass during the green leaf stage and seasonal changes in whole-tree N content per plant tissue of *Fagus crenata* Blume (*n* = 4) with ± denoting SD.

Tissue	Biomass [kg]	N [g N]		
		Green leaf stage	Pre-abscission stage	Post-abscission stage
roots	117 ± 9[a]	679 ± 53	862 ± 119*	893 ± 106*
sapwood	362 ± 20[b]	245 ± 70	208 ± 84	339 ± 126*
branches	68 ± 5[a]	54 ± 8	52 ± 5	83 ± 10*
leaves	8.7 ± 0.1[c]	193 ± 20	144 ± 26*	64 ± 6*

a = Calculated following Ono et al. (2013)
b = stem weight calculated following Ono et al. (2013). Then, from stem weight, sapwood weight was calculated following Gebauer et al. (2008).
c = Calculated following Tateishi et al. (2010).
* = significant differences among same tissues in comparison to green leaf stage

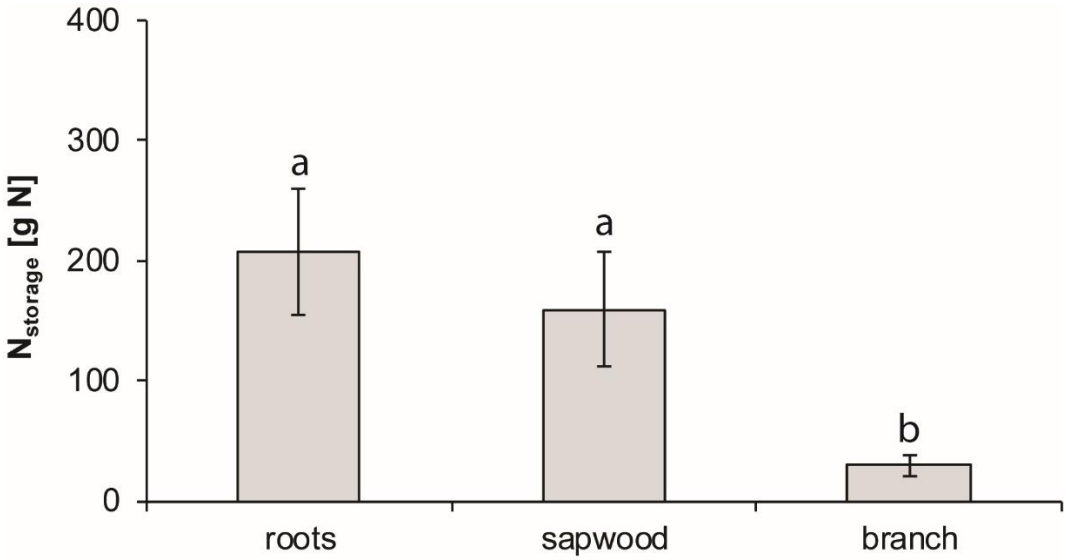


Figure 4: Total N storage of coarse roots, sapwood, and branches of *Fagus crenata* Blume. The error bars denote SD (n = 9), $p < 0.01$.

4 Discussion

4.1 Reabsorption, Storage, and Remobilization of N in Plant Tissues

The N cycle in *Fagus crenata* followed a well-established pattern: in the green leaf stage, most N was bound in leaves' photosynthetic apparatus (Ueda e al., 2011). In the pre-abscission stage, leaf N was reabsorbed until leaf abscission. Subsequently, fresh litter in the post-abscission stage was depleted in N content (*RE* 66%). Enta et al. (2019) and Yasumura et al. (2005) reported a similar value (*RE* 68-71%) for this species. Subsequently, N was stored in a sequential temporal pattern: first, it was stored in coarse roots during the pre-abscission stage, followed by N storage in the sapwood in the post-abscission stage. In previous studies (Millard et al., 2001; Warren et al., 2003; Millard et al., 2010; Ueda et al., 2011), this temporal pattern of N storage in coarse roots and sapwood of deciduous trees has not been shown. In the post-abscission stage, when most N was stored, roots tended to store more N (ns) than sapwood. N reabsorption from leaves contributed 32% to overall N storage in the post-abscission stage.

During the shoot growth stage, N contents peaked in roots (ns) and sapwood ($p < 0.01$). Together with soil N taken up in late April, stored N supported shoot growth in the shoot growth stage (Gessler et al., 1998) despite heavy snow cover, as is typical for this region. The continuous uptake of soil N during the dormant phase of the tree in the post-abscission stage could also contribute to the high N content found in the shoot growth stage. However,

N uptake during dormancy has been reported to be minimal and only few studies have found it for boreal species during warm periods of the winter season season (Ueda et al., 2011; Cooke and Weih, 2016). Tree internal N storage strongly determines the beech masting period (Dyckman & Flessa, 2001; Han et al., 2014), and thus, the quantification of N uptake during the dormant period of this species needs to be explored in more detail. In the green leaf stage, after stored N was remobilized to support new shoot and leaf growth, coarse roots and sapwood were left depleted (Ueda et al., 2011; Wyka et al., 2016). We did not quantify the contribution of stored N to new growth, but Dyckman & Flessa (2001) reported that previous-year N contributes to leaf N with about 15% in *Fagus sylvatica*.

4.2 Effect of Soil N Uptake on Plant $\delta^{15}N$

Soil total N content, as well as inorganic N content, decreased with depth as $\delta^{15}N$ became heavier due to the input of fresh N on the soil surface: precipitation and microbial turnover of litter, as well as illuviation of ^{15}N-rich materials to deeper soil layers, are the main mechanisms (Koba et al., 1997; 1998; Shi et al. 2014; Zhou et al. 2014). Tateno et al. (2005) found that foliar $\delta^{15}N$ of the green leaf stage depends on ammonium and nitrate availability. In this latter study, *Fagus crenata* trees on upper slopes took up ammonium due to a lack of nitrate, leading to higher foliar $\delta^{15}N$ values (−1.0‰) than at the bottom of the slope, where nitrate was more abundant, leading to ^{15}N depletion in leaves (−2.0 ‰). In our study, we found a high availability of ammonium and a negligible amount of nitrate, which explained the $\delta^{15}N$ value of −1.2 ± 0.6‰ in agreement with Tateno et al.'s (2005) findings for trees in upper slope positions. Additionally, we measured low potential mineralization rates (1mg kg^{-1} soil in 30 days, data not published) in a nearby *Cryptomeria japonica* stand being similar to Tateno et al.'s (2005) results. Thus, we concluded that our trees rather relied on ammonium rather than nitrate, although under different conditions, they would preferably acquire nitrate (Rennenberg & Dannenmann, 2015; Dannenmann et al., 2016), but climatic conditions and the acidic pH strongly limited the nitrification process.

The inorganic soil N taken up by tree roots and mycorrhiza undergoes isotopic fractionation as mycorrhizal fungi selectively accumulate ^{15}N and thus, a rather depleted ^{15}N fraction passes to the host plants (Tateno et al., 2005; Högberg et al., 2011; Hobbie & Högberg, 2012; Clemmensen et al., 2013). Leberecht et al. (2016) demonstrated that the ectomycorrhizal fungi (EMF) associated with *Fagus sylvatica* preferably uptake ammonium from the soil,

resulting in a more enriched ^{15}N accumulation in the EMF in comparison to EMF acquiring nitrate. In both cases, $\delta^{15}N$ values of the host plants will be lower, but the depletion in ^{15}N will be less pronounced for N deriving from ammonium. This suggests a stronger control of N supply to the host tree by the EMF acquiring ammonium than would be the case with EMF taking up nitrate. Soil $\delta^{15}N$ was 5‰ greater than root $\delta^{15}N$, revealing the effect of microbial turn over and uptake of inorganic N via mycorrhiza (Hobbie & Högberg, 2012).

4.2 Effect of Intra-Plant N Movement on $\delta^{15}N$

In general, leaves' $\delta^{15}N$ ratio falls in line with the findings of Tateno et al. (2005), who found similar values for *Fagus crenata* in lower slope positions under similar soil conditions. In the pre-abscission stage, leaves were significantly enriched in ^{15}N by 0.5‰. At the same time, coarse roots' $\delta^{15}N$ tended to decrease (ns) by 0.5‰. Depleted reabsorbed leaf N seemed to be transported and stored in coarse roots during the pre-abscission stage, possibly together with taken up soil N, forming the bulk of coarse roots' N storage. During the post-abscission stage, N storage only increased marginally (ns); however, a significant decrease in coarse roots' $\delta^{15}N$ suggests that only ^{15}N-depleted soil N was stored, since the reabsorption of leaf N was already concluded.

After leaf abscission, N content significantly decreased in litter, depleting the $\delta^{15}N$ value by 1.5‰. Thus, shortly before leaf senescence, ^{15}N-enriched N was reabsorbed and subsequently stored in sapwood, increasing it by 1.5‰. In contrast to the results of Kolb & Evans (2002),we found N isotope fractionation during reabsorption in leaves.

Thus, three phases of N storage could be observed:

(1) ^{15}N-depleted N was reabsorbed from leaves to coarse roots in the pre-abscission stage, which was followed by (2) ^{15}N-enriched leaf N reabsorption, significantly enriching sapwood in the heavier isotope during the post-abscission stage. (3) At the same time, coarse roots' $\delta^{15}N$ decreased, suggesting a strong N isotope fractionation in the EMF during the uptake and storage of soil N, as it occurs under ammonium-rich soil conditions (Leberecht et al., 2016). In the transition from the post-abscission to the shoot growth stage, coarse roots were enriched in ^{15}N, while sapwood was depleted. This suggests a movement of depleted N from coarse roots to sapwood, which eventually would be used for new shoot growth and leaf production. Only during the green leaf stage did all measured tissues show similar $\delta^{15}N$

values. Further analysis is necessary to confirm whether foliar $\delta^{15}N$ during the green leaf stage could be used as a proxy for whole-tree $\delta^{15}N$ in other tree species, as has been suggested in previous studies (Kolb & Evans, 2002).

4.3 Plant Tissues Free Amino Acids

During the green leaf stage, free amino acid content (FAAC) was generally low in all tissues. However, coarse roots' FAAC was the highest among all tissues, possibly because of soil N uptake (Finzi & Berthrong, 2005). DON represents a major soluble N pool in the soil and is mostly composed of amino acids, which can be directly taken up by roots (Jones et al., 2004). In our study, DON made up 21% of all soluble N of the top 30 cm. Roberts & Jones (2012) reported that DON made up about 40% of all plant available N in a eutric cambisol, with free amino acids accounting for 10%. The low amino acid concentration in the soil solution contributed to the extremely fast rate at which these are removed from the soil, thus explaining the high values found in coarse roots of our study.

During the pre-abscission stage, FAAC increased sharply in leaves as a result of protein hydrolysis (Warren et al., 2003). During the post-abscission stage, as leaf N reabsorption ended, a sharp decrease in total N and FAAC content in the fresh litter was observed, corresponding with significant increases in sapwood and coarse roots. This was likely linked to the continuous uptake of soil N (Finzi & Berthrong, 2005) and further storage during the dormant phase of trees in the post-abscission stage. FAAC was high in all plant tissues during the shoot growth stage, suggesting that in coarse roots, N was internally remobilized within the tree and also absorbed from the soil. N is transported via sapwood to the emerging leaves to synthesize proteins for building cell walls and the photosynthetic apparatus (Ueda et al., 2011).

In coarse roots, the most abundant free amino acid throughout the phenological stages was asparagine. Asparagine is known to act as a transport and storage form of N in plants (Fotelli et al., 2002) and its peak in the shoot growth stage revealed its high importance for N remobilization in spring, as has also been found in the case of apple trees (Malaguti et al., 2001). Other major compounds were alanine and arginine. Alanine is closely connected with N metabolism (Miyashita et al., 2007) and is commonly found under stress conditions (e.g. temperature stress), yet its precise function is unclear. As alanine is high in all tissues in the shoot growth, as well as in the pre- and post-abscission stage, it might be linked not only to

temperature stress caused by the long snow cover in our plots, but also to the transport of N. Arginine seemed to be linked to stress and transport as well, as it contributes to building and degrading proteins (Fotelli et al., 2002), which coincides with its occurrence during the shoot growth and pre-abscission stage.

Similar patterns were found in the sapwood for asparagine and alanine being linked to N transport during remobilization and storage. Although only low contents of glutamic acid and glutamine were found, these two amino acids are also associated with the transport and storage of N (Malaguti et al. 2001). The occurrence of proline in the shoot growth stage was likely linked to snow-related temperature stress (Verbruggen & Hermans, 2008). Finally, leaves predominantly contained alanine, which largely occurred during the shoot growth and pre-abscission stage, supporting our findings of its transport function linked to N storage and remobilization.

4.4 Correlation between Free Amino Acid Content and Plant Tissues $\delta^{15}N$

We observed four synchronized events between FAAC variation and $\delta^{15}N$ fractionation in leaves. (1) In the pre-abscission stage, free amino acids are released after protein hydrolysis (Warren et al., 2003) and fractionation of ^{15}N occurred. (2) In the post-abscission stage, as all the released amino acids were reabsorbed from leaves, $\delta^{15}N$ significantly decreased. (3) During the shoot growth stage, ^{15}N increased in leaves and leaf FAAC increased in comparison to litter. (4) During the green leaf stage, FAAC was low, as free amino acids were synthesized into proteins (Ueda et al., 2011), and leaf $\delta^{15}N$ showed no variation.

Therefore, the seasonal increase in leaf FAAC appears to be the reason for the seasonal enrichment of leaf ^{15}N. However, a decrease in FAAC, as observed during the green leaf and post-abscission stage, does not automatically mean a depletion in leaf ^{15}N. A decrease in leaf $\delta^{15}N$ in the green leaf stage was not observed because the free amino acids were bound in proteins and are present in the leaf (Näsholm, 1994). In contrast, in the post-abscission stage, the small leaf FAAC reflects their absence because they have been reabsorbed to storage tissues. The single most abundant free amino acid in leaves was alanine, which suggests that the seasonal variability of this amino acid is mainly responsible for leaf $\delta^{15}N$ fractionation. Singling out seasonal measurements of alanine $\delta^{15}N$ during the pre-abscission stage could clarify its contribution to leaf ^{15}N fractionation (Chikaraishi et al., 2009). We found a less clear connection between FAAC and $\delta^{15}N$ in sapwood and coarse roots, since

the source of stored N originates from leaf N reabsorption and soil N uptake, as reflected by the difference in free amino acids' composition in these tissues (Näsholm & Ericsson, 1990) resulting in a lower adjusted R^2 in the linear regression analysis. However, we could link root and sapwood asparagine content to cause an enrichment in $\delta^{15}N$. In contrast to leaves and roots where FAAC were found in abundance, sapwood seemed to be dominated by other N-containing compounds, e.g., linked to the incomplete breakdown of leaf N storage to free amino acids (Kolb & Evans, 2002; Näsholm & Ericsson, 1990). Future studies should include measurements of protein in order to increase the R^2, especially in the case of sapwood.

5 Conclusions

The results obtained in this study showed that, on average, adult *Fagus crenata* trees had an N reabsorption efficiency of 66%. The reabsorbed foliar N was sequentially stored in coarse roots during the pre-abscission stage and in the sapwood in the post-abscission stage, from where it was remobilized to growing tissues during the shoot growth stage. Leaf N made up 32% of the total storage in roots and sapwood, making leaf N reabsorption vital for the species' survival. Nitrogen was evenly stored between coarse roots and sapwood. N isotope fractionation occurred when N reabsorption took place before leaf abscission. First, ^{15}N-depleted N from leaves was traced to ^{15}N-depleted N stored in coarse roots, followed by the reabsorption of ^{15}N-enriched N that was traced to ^{15}N-enriched N stored in sapwood. Free amino acids' dynamics could partially explain changes in $\delta^{15}N$ in all measured tissues.

Chapter 4

Seasonal whole-tree Nitrogen movement in *Larix kaempferi* and *Robinia pseudoacacia* revealed by plant tissues N isotopic composition and free amino acids content

Felix Seidel[1], M. Larry Lopez C.[1], Luisella Celi[2], Eleonora Bonifacio[2], Akira Oikawa[1], Toshiro Yamanaka[3]

[1]*Faculty of Agriculture, Yamagata University, Tsuruoka, Japan*

[2]*Department of Agricultural, Forest and Food Sciences, Università degli Studi di Torino, Torino, Italy*

[3]*Tokyo University of Marine Science and Technology, Tokyo, Japan*

Author for correspondence:

Felix Seidel

Tel.: +81-235-28-2961

E-Mail: seidel.geo-science@gmx.de

Faculty of Agriculture Yamagata University

Tsuruoka-shi, Wakabamachi 1-23, 997-8555

Yamagata Prefecture, Japan

Abstract

Background and Objectives: Larix species distribute extensively in boreal and temperate regions and leguminous *Robinia pseudoacacia* is usually an invasive species commonly found in temperate forests in the northern hemisphere. Recent studies of leaf N isotopic composition for these two species have shown similar values and seasonal variability despite their taxonomically contrasting characteristics. The objective of this study was to clarify the reasons for this similarity by tracing and quantifying N movement linking free amino acids with $\delta^{15}N$ fractionation at the whole tree level in both species.

Materials and Methods: N content, isotope ratio and free amino acids content were measured in fine roots, coarse roots, sapwood, leaves, and litter along the growing season in eight adult *Larix kaempferi* and five *Robinia pseudoacacia* trees.

Results: Nitrogen was released from storage tissues during the shoot growth stage, which was correlated with changes in free amino acids content. Shortly before leaf senescence, nitrogen was reabsorbed and stored in the woody parts of both species. *Larix* predominantly stored soil N, while *Robinia* mainly stored reabsorbed leaf N as suggested by whole-tree measurements and $\delta^{15}N$. *Robinia* reabsorbed leaf N earlier than *Larix*. During resorption, N was stored first in the coarse roots and then in the sapwood, which is an agreement with free amino acids transport and storage

Conclusions: We concluded that the temporal storage pattern of N first in the coarse roots followed by the sapwood might be a general pattern for trees of the cold temperate zone. Further, stable isotopic analysis in combination with free amino acid content and whole-tree measurements proved to be useful tools to efficiently trace movement of N additionally elucidating the reasons why leaf $\delta^{15}N$ of both species was similar although their N sources differ. It was not possible to link changes in free amino acids content with $\delta^{15}N$ fractionation due to either the lack of fractionation (*Larix*) or the lack of changes in amino acids content (*Robinia*).

Keywords: Amino acids, Leguminous, Nitrogen recycling, Nitrogen storage, Nitrogen stable isotopes, Whole-tree N

1 Introduction

Larch species are broadly distributed in the northern hemisphere, making up more than one-third of the Eurasian boreal forest being most common in East Asia (Choi et al., 2008). *Larix* is a genus planted in many parts of the world as these trees are appreciated for their good mechanical properties and high natural durability of the heartwood forming about 60 - 80% of the total stem diameter depending on tree age. The formation of heartwood is determined by biotic and abiotic factors influencing canopy size (e.g. competition, thinning, pruning) or soil conditions promoting or limiting growth (e.g. irrigation, fertilization) (Pâques, 2000). Heartwood shows no temporal variation in nitrogen (N) content throughout the growing season, as N is relocated with the flow of water which moves in the sapwood (Tomlinson et al., 2014). Thus, sapwood is the only part of the stem being able to store and remobilize N. Prior to leaf abscission, N is reabsorbed and stored in sapwood and roots. This N will be remobilized in the shoot growth stage of the next year contributing greatly to growth of new tissues (Millard & Grelet, 2010; Han et al., 2014). So far, in situ studies of temporal N partitioning and movement in adult trees throughout all four seasons on whole tree level have been scarce. Even more so for *Larix kaempferi* as it is a tree growing under deep snow cover during winter months (Fukushima et al, 1995) limiting access to the sites especially and making sampling elaborate.

The standard method of tracing the intra-plant movement of N is the analysis of N stable isotope ($\delta^{15}N$) allowing the identification of N sources-sink intra-plant N transformations (Kolb & Evans, 2002; Tateno et al, 2005; Lopez et al. 2010, 2014). A study by Enta et al. (2019), found that deciduous coniferous *Larix kaempferi* and deciduous broad-leaved leguminous *Robinia pseudoacacia* showed similar leaf $\delta^{15}N$ values. This was peculiar as their N source and thus most likely their $\delta^{15}N$ value is poles apart: *Larix kaempferi* uptakes N from the soil and *Robinia pseudoacacia* fixes it from the atmosphere. The reason for these findings could not identified in that study, as only measurements of leaves during different phenological stages were analysed. *Robinia pseudoacacia* is a nitrogen fixing invasive species and the second most abundant deciduous tree species in the world (Malcom et al., 2008) elevating soil N concentrations and increasing the speed of soil N cycling and soil $\delta^{15}N$ values (Rice et al. 2004; Malcom et al., 2008; Lopez et al., 2014). Analysing different plant tissues of both species along different phenological stages and the soil of these stands could shed light

on the reasons of the similarity in leaf $\delta^{15}N$ values of *Larix kaempferi* and *Robinia pseudoacacia*.

In addition, N movement can be detected by changes in free amino acids content in plant tissues, as they transport N within the tree (Näsholm & Ericsson, 1990; Pietilä et al., 1991; Warren et al., 2003; Nordin et al., 2001; Xu & Xiao, 2016). By combining $\delta^{15}N$ measurements with the quantification of free amino acids content, it may be possible to gain a deeper understanding of the N cycling of *Larix kaempferi* and *Robinia pseudoacacia*. These tools have already been proven useful in food webs of marine and terrestrial organisms (Chikaraishi et al, 2009 and 2011), as well as in plant tissues of *Cryptomeria japonica* (Seidel et al., 2019a) and *Fagus crenata* (Seidel et al., 2019b). In the latter study, movement of free amino acids could be directly linked to a change in the isotopic composition of senescing leaves. Additionally, a distinct temporal pattern of N storage first in the roots during the pre-abscission stage, followed by storage of N in the sapwood during the post-abscission stage was revealed for evergreen coniferous *Cryptomeria japonica* and broad-leaved *Fagus crenata,* which has not been shown in previous studies (Millard et al., 2001; Warren et al., 2003; Millard & Grelet, 2010; Ueda et al., 2011).

Thus, in this study, we hypothesized that N movement could be tracked with $\delta^{15}N$ and its fractionation could be explained by changes in tissues free amino acids content subsequently explaining why leaf tissues of both species showed similar $\delta^{15}N$ values. It would provide additional insights into two of the most distributed and planted tree species around the world and contribute to the interpretation of leave $\delta^{15}N$ for large scale studies.

Our objectives were (1) to assess whole tree N partitioning in four phenological stages (shoot growth, green leaf, pre- and post-abscission), (2) to determine plant tissues fractionation of $\delta^{15}N$ pattern in both species that lead to the similarities in leaf $\delta^{15}N$ of these two species and finally, (3) if changes in free amino acid content affect $\delta^{15}N$.

2 Materials and Methods

2.1 Study site

The study site is situated in north-eastern Japan in Yamagata prefecture on the Japanese seaside. The climate is humid, with an annual precipitation of 3000 mm: approximately half of it corresponds to snow, covering the sampling sites from December to the end of April at an elevation between 400 m and 650 m above sea level. The driest month is June with approximately 90 mm of rain and the wettest is December with 600 mm. Nitrogen deposition with precipitation is low, i.e. 10-15 kg TN ha^{-1} $year^{-1}$ (e.g. Kanto region 30-38.5 kg TN ha^{-1} $year^{-1}$) (Watanabe et al., 2012).

For *Fagus crenata*, eight trees in three sampling sites (two sites with three trees, one site with two trees) were chosen, distributing within a distance of 1.5 km in the Research Forest of Yamagata University. The sites were selected by accessibility throughout the year, stand age and purity. The three sites were in a mixed forest, which was dominated by *Larix kaempferi* (~80-90%). For *Robinia pseudoacacia*, five trees were sampled in a single stand, as it was the only location in the Yamagata University Research Forest where it grows in a species uniformity stand. To ensure comparability of the sites, prior soil samplings were conducted to ensure comparability of the sites in terms of soil type and nutrient status.

2.2 Sample collection and treatments

Eight *Larix kaempferi (*Lamb.) trees of similar age (40-50 years old) as well as five *Robinia p* Soil samples were collected on August the 7, 2017 from soil pits close to the selected trees. Soil horizons were identified in the field following the FAO guidelines (FAO, 2006). Genetic horizons were sampled for soil characterization, while samples for nitrogen and $\delta^{15}N$ analysis were taken at fixed depths from 0-5 cm, 5 -15 cm and 15-30 cm from three pits as replicates, surrounding each individual tree. *seudoacacia* (L.) (7 years old) were chosen for sampling (Table 1). All samples were transported in plastic bags after collection in the field, air-dried, sieved (< 2 mm), and stored until analysis. The samples used for the determination of inorganic N were frozen after transport, and stored in the dark. Before analysis, they were thawed overnight in a refrigerator and sieved (< 2 mm).

Table 1: Characteristics of sampled *Larix kaempferi* Lamb. (n = 8) and *Robinia pseudoacacia* L. (n=5).

Site	Coordinates	m a.s.l.	Tree	DBH	Height	Age
Larix 1	N38° 33.427' E139° 52.560'	650	A	42.3	25.9	48
			B	38.9	21.1	45
			C	35.6	24.8	41
Larix 2	N38° 33.433' E139° 52.513'	610	A	38.1	27.4	40
			B	36.3	23.7	43
			C	33.7	20.4	40
Larix 3	N38° 33.469' E139° 52.433'	540	A	49.3	31.8	50
			B	34.6	24.5	45
Robinia	N38° 33.560' E139° 52.125'	400	A	10.7	10.1	7
			B	10.9	12.5	7
			C	13.2	12.9	7
			D	15.7	14.2	7
			E	20.1	13.0	7

DBH= diameter at breast height

In 2017, coarse roots (> 2 mm), fine roots (< 2 mm), sapwood and leaf samples were taken on May 16 (shoot growth stage), August 3 (green leaf stage), October 19 (pre-abscission stage), and all except fine roots (as they die during winter) on November 29 (post-abscission stage) with fresh litter collected from litter traps (size: 1 m², placed on September 4). Fine and coarse roots of single trees were dug out, identified and cut from the trunk up to a depth of 30 cm. Three samples surrounding each tree were taken and pooled. Sapwood was sampled by using a 10 mm increment borer. Heartwood was not sampled, assuming that there was no variation in N content throughout the growing season. This was confirmed by three test samples of heartwood taken in summer 2018. Leaf samples were taken from the lower canopy as there seems to be no significant difference in leaf N content depending on canopy position (Son & Gower, 1992). By sampling different leaf clusters, we included possible variations in N content.

All samples were transported in plastic bags and oven dried in the laboratory. All samples were dried at 40°C for at least 48 hrs. Subsequently, the samples were ground and stored until analysis.

2.3 Soil analysis

Soil texture was measured with a laser diffraction particle size analyzer (Coulter LS200 with an attached Fluid Module, Beckman Coulter GmbH, Germany), after treating the samples with H_2O_2 and $Na_4P_2O_7$. The analyses were triplicated. The pH was determined potentiometrically in a 1:2.5 soil : water suspension. The carbon (C) and N contents were analysed by dry combustion using SUMIGRAPH NC-220F automatic high sensitive NC analyzer SCAS (Japan). The results were expressed on a dry-weight (105°C) basis.

Total soluble N (TN_b) was extracted from the soil samples by shaking them with 50 ml of 1M KCl. The supernatant was centrifuged, filtered (0.45 µm), stored in a refrigerator and analysed with a TOC/TN_b analyser (vario TOC cube, elementar, Germany) for TNb determination. Ammonium content was determined using the method of Crooke et al. (1971), whereas nitrate content was determined by using the method of Mulvaney (1996) modified by Miranda et al. (2001). The colorimetric determination of ammonium and nitrate content was conducted with a U-2000 Spectrophotometer (Hitachi, Japan). Dissolved organic nitrogen (DON) expressed in g kg^{-1} was calculated as follows:

$$DON = TN_b - (NH_4^+ + NO_3^-)$$

2.3.1 Total Nitrogen and $\delta^{15}N$ Isotope analysis

For the determination of total N content and $\delta^{15}N$ in plant tissues, a Thermo Quest EA1110 Elemental Analyzer (Italy) connected to an IsoPrime (GV Instruments, UK) was used. The isotopic compositions of samples were expressed relative to atmospheric N_2 ($\delta^{15}N$ = 0‰) on scales normalized to the known $\delta^{15}N$ values of laboratory working standards for glycine ($\delta^{15}N$ = −0.3), which was normalized to L-glutamic acid distributed as USGS-40 ($\delta^{15}N$ = −0.2‰) by SI Science Inc., Japan. Additionally a tertiary reference material was used, namely the in-house laboratory standard acetanilide (−0.89‰). The three working standards were analysed after every eight to ten samples during CF-IRMS runs to assess the replicability of the isotope measurements and normalization. One pulse of pure N_2 reference gas from a tank reservoir ($\delta^{15}N$ = −2.5‰) was discharged into the IRMS at the beginning of each chromatogram for both

standards and samples. The accuracy obtained for standards and samples during the overall analytical procedure was better than ±0.2‰.

The results of $\delta^{15}N$ were expressed as ‰ deviation, relative to atmospheric N_2 ($\delta^{15}N$= 0 ‰):

$$\delta^{15}\mathrm{N} = \left(\frac{(^{15}N/^{14}N)_{sample}}{(^{15}N/^{14}N)_{standard}} - 1 \right)$$

2.3.2 Amino acid analysis

Amino acids were extracted from the dried powders of plant materials with methanol and separated by liquid/liquid extraction with ultracentrifugation. Nineteen amino acids (Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val) were quantified by capillary electrophoresis mass spectrometry (CE-MS). The preparation conditions during sample extraction and CE-MS analysis followed Oikawa et al. (2015). Only values over the detection limit were presented (Appendix 1).

2.3.3 Whole-tree N calculations

For *Larix kaempferi*, whole-tree biomass was calculated as follows:

For stem and branch weight, the allometric equation by Hosoda & Iehara (2010) for *Larix kaempferi* was used.

$$W = a * D^b * H^c$$

Where *W* is the biomass [kg], *D* the tree diameter at breast height (DBH) [cm], and H the height of a tree [m]. Further, *a*, *b*, and *c* are coefficients gained from linear regression (a= 0.017872, b= 1.801722 and c= 1.171699 for stem and a= 0.092742, b= 2.905918 and c= -1.288063 for branches).

Further, from the calculated stem weight, sapwood weight was estimated assuming that 22% of all stem weight is sapwood as reported by Pâques (2001) for Japanese larch and verified with tree core sampling on all measured trees.

For roots no allometric equations could be found but Sakai et al. (2007) reported that for *Larix kaempferi* the trunk/root ratio is 4:1 thus, we used the total stem weight obtained from equation (1) and divided it by 4. Further, we did not estimate fine root biomass, as its weight seems negligible in comparison to coarse roots (Chenlemuge et al., 2013).

Hosoda & Iehara (2010) reported that leaf biomass determination had a low precision thus, for leaf biomass estimation the values obtained from litter traps [1 m²] were scaled up to whole-tree canopy area by measuring whole tree stand size [m²]. Stand size was calculated by measuring canopy base diameter in four directions for each tree.

For *Robinia pseudoacacia*, three whole trees were cut in the green leaf period of 2018, to destructively collect biomass data of leaves and sapwood. Before cutting the trees, branches were cut off to reduce leaf loss through the falling stem. Leaves were dried at 50°C before weighing to estimate whole-tree leaf weight. Every two meters a tree disc was cut from stems, dried at 50°C and separated into heart- and sapwood sections. Based on the disks characteristics, the volume and weight of the sapwood was calculated. For the root weight, a general biomass equation model based on tree diameter a by Blujdea et al. (2012) for *Robinia pseudoacacia* was applied.

$$\ln M = \ln a + b \ln x$$

Where *M* is the biomass [kg], *a* and *b* are coefficients gained from linear regression (a= 3.6264, b= 2.0413) and *x* is the DBH [cm]. We confirmed the reliability of this equation by comparing our results from the stem and leaf measurements with the results generated by the equation above for these plant tissues and found comparable results. For litter weight, we followed the same methodology described for *Larix kaempferi* using litter traps and scaling up to whole-tree canopy area.

Furthermore, we assumed that in the post-abscission stage all N was in the respective storage tissues (coarse roots and sapwood) while storage was considered empty during the green leaf stage since N is needed in the growing tissues of both tree species. Thus, whole-tree N storage was calculated as follows:

$$N_{storage} = N_{post} - N_{green}$$

Where $N_{storage}$ is the N stored in a certain tissue, N_{post} is the N content during the post-abscission stage and N_{green} is the N content during green leaf stage. We calculated this value for all plant tissues as relative amounts (g N kg^{-1}) and as whole-tree absolute N storage (g N).

Finally, reabsorption efficiency (*NRE*) was calculated as:

$$NRE = \left(1 - \frac{mass\ of\ N\ in\ senesced\ leaves}{mass\ of\ N\ in\ greeen\ leaves}\right) \times 100$$

Nitrogen was expressed as mass per leaf dry mass (mg g^{-1}). Litter, represents the senesced leaves in November, while green leaf is the matured leaf in August. We calculated whole-tree total reabsorbed leaf N by considering the total tree leaf weight scaled up from litter trap measurements. Further, we calculated NRE* to account for leaf mass loss during N reabsorption (Factor 0.754 for conifers) following Vergutz et al. (2012), which could lead to an underestimation of 10% (van Heerwaarden et al., 2003). Finally, nitrogen resorption proficiency (NRP) was calculated. It represents a more stable indicator of plant ability to recycle nutrients than NRE. Nitrogen content per leaf mass in senescing leaves was used as NRP (Killingbeck, 1996; Yasumura et al. 2005; Yuan et al. 2005). Leaves that can reduce N concentration to a lower level are more proficient in resorbing N. The NRP is expressed in % dry mass.

2.3.4 Statistical analysis

One-way ANOVA was applied to determine the statistical significance of differences in nitrogen content, $\delta^{15}N$ and amino acids content. If significant differences were found, post-hoc multiple comparison was subsequently conducted, using Tukey-Kramer test at the significance levels of 0.05 and 0.01.

3 Results

3.1 Soil characteristics

The soils in all sites were identified as brown forest soils (Cambisols) with an average depth of 70 cm and a sandy-loamy texture. In the top 30 cm of the *Larix kaempferi* sites, we found a pH of 4.3 and a C:N ratio of 15, on average. Soil total N content decreased with depth by 60% as well as total soluble N content, which accounted for approximately 5% of the total N. The majority of soluble N was made by organic compounds (DON, ca. 90%), and the concentration of ammonium was 10-fold that of nitrate. $\delta^{15}N$ was instead 3 times higher in the bottom layer than in the top one ($P < 0.01$) (Table 2). The pH in the *Robinia pseudoacacia* site was significantly higher (5.5) as well as the total N content, while the C:N ratio, isotopic composition and soluble N were similar between sites. Soil total N content decreased with depth by 80%, soluble N decreased by 50% and was made by organic compounds (DON, ca. 70 %).The concentration of nitrate was 4-fold that of ammonium in the soil of the *Robinia pseudoacacia* site.

Table 2: Soil characteristics with ± denoting SD. Significant differences among same parameters are highlighted in bold.

Depth [cm]	pH [-]	Total N [g N kg^{-1}]	C:N	$\delta^{15}N$ [‰]	TNb [g N kg^{-1}]	Ammonium [g N kg^{-1}]	Nitrate [g N kg^{-1}]	DON [g N kg^{-1}]
0-5	4.2 ± 0.6	3.5 ± 2.6	17 ± 7	0.9 ± 1.5	0.14 ± 0.06	0.01 ± < 0.01	0.001 ± 0.001	0.13 ± 0.05
5-15	4.4 ± 0.4	1.8 ± 0.9	15 ± 5	2.0 ± 1.5	0.09 ± < 0.01	< 0.01 ± < 0.01	0.001 ± < 0.001	0.08 ± 0.01
15-30	4.4 ± 0.2	**1.4 ± 0.4****	14 ± 6	**3.1 ± 1.7****	**0.07 ± < 0.01***	<0.01 ± < 0.01	< 0.001 ± < 0.001	0.08 ± 0.01

* P < 0.05 ** = P< 0.01

3.2 Total nitrogen content & $\delta^{15}N$ Isotope analysis in tree tissues

Fine roots N content of *Larix kaempferi* did not vary along the phenological stages and contained twice as much N in comparison to coarse roots ($P < 0.01$). Coarse roots N content decreased ($P < 0.01$) from the shoot growth to the green leaf stage by 40% followed by an increase ($P < 0.01$) of 50% during the pre-abscission stage. There was no change in coarse roots N content in the post-abscission or shoot growth stage (Fig.1a). Fine roots N content of

Robinia pseudoacacia decreased ($P < 0.05$) by 30% from the shoot growth to the green leaf stage and was 20% to 50% higher ($P < 0.01$) than coarse roots N content (Fig.2a). *Robinia pseudoacacia* coarse roots N content followed the same pattern as *Larix kaempferi* with a more pronounced decrease from the shoot growth to the green leaf stage (50%). Generally, root N content of *Robinia pseudoacacia* was 80% higher than that of *Larix kaempferi* roots.

Sapwood N content of *Larix kaempferi* was the highest in the shoot growth stage ($P < 0.01$) and decreased ($P < 0.01$) by 50% at the green leaf stage with no change during the pre-abscission stage. In the post-abscission stage, sapwood N content increased ($P < 0.01$) by 45% (Fig.1b). Sapwood N content of *Robinia pseudoacacia* did not change significantly from the shoot growth to the pre-abscission stage but increased ($P < 0.01$) during the post-abscission stage by 30% and was 75% higher ($P < 0.01$) than in *Larix kaempferi* (Fig.2b).

Leaf N content of *Larix kaempferi* was highest in the shoot growth stage ($P < 0.01$) and decreased by 30% from the shoot growth to the green leaf stage followed by another decrease of 40% from the pre-abscission to the post-abscission stage (Fig.1c). The NRE was 36% while NRE* was 52% and NRP 0.9. Leaf N content of *Robinia pseudoacacia* was also the highest in the shoot growth stage and decreased ($P < 0.05$) by 20% during the pre-abscission period, followed by an additional decrease ($P < 0.01$) of 20% after leaf abscission (Fig.2c). Generally, leaf N content of *Robinia pseudoacacia* was 98% higher ($P < 0.01$) than leaves of *Larix kaempferi*. Similar to *Larix kaempferi*, the NRE was 32% while NRE* was 47% while the NRP of 2.6 was considerably lower in *Robinia pseudoacacia*.

For *Larix kaempferi* roots biomass was the heaviest of all the plant tissues followed by sapwood, branches and leaves (Table 3). N stored in coarse roots stored nearly 80% of all stored N, while sapwood and branches stored 15% and 5% respectively. Whole-tree reabsorbed leaf N made up 7 % of all stored N. For *Robinia pseudoacacia*, sapwood was the heaviest plant tissue followed by roots, branches and leaves (Table 3). Coarse roots stored about 90% of all stored N, while sapwood and branches stored in total 10%. Whole-tree reabsorbed leaf N made up 75% percent of all stored N.

All measured plant tissues of *Larix kaempferi* showed no variation in $\delta^{15}N$ throughout the four phenological stages with coarse roots as the exception. During the green leaf stage coarse roots were significantly lighter than in all other phenological stages. Leaf $\delta^{15}N$ values were the lightest followed by sapwood, fine roots and coarse roots (Fig.1). Plant tissues of

Robinia pseudoacacia displayed variation in $\delta^{15}N$ in coarse roots and leaves but not in sapwood and fine roots (Fig. 2). Coarse roots $\delta^{15}N$ became 1‰ lighter ($P < 0.01$) from shoot growth to the pre-and post-abscission stage. On the other hand, leaves isotopic composition became gradually heavier from the green leaf ($P < 0.05$) to the post-abscission stage ($P < 0.01$).

Along all phenological stages, $\delta^{15}N$ values of fine roots, sapwood and leaves of both tree species were similar, while the $\delta^{15}N$ of coarse roots differed ($P < 0.05$) with *Larix kaempferi* showing generally heavier $\delta^{15}N$ values. During the green leaf stage, both species $\delta^{15}N$ values were similar.

Table 3: Biomass during the green leaf stage and seasonal change in whole-tree N content and storage per plant tissue of *Larix kaempferi* Lamb. (n=8) with ± denoting SD. Significant differences among same plant tissues are highlighted in bold.

Tissue	Biomass [kg]	N [g N]			Stored N [g N]
		Green leaf stage	Pre-abscission stage	Post-abscission stage	
roots	133 ± 38[a]	500 ± 150	**720 ± 183***	**759 ± 127***	260 ± 91
sapwood	112 ± 31[b]	222 ± 61	264 ± 68	**392 ± 116****	48 ± 18
branches	55 ± 9[c]	31 ± 6	37 ± 6	**54 ± 11****	23 ± 6
leaves	4 ± 1[b]	64 ± 15	65 ± 12	**40 ± 10****	24 ± 5 g leaf N reabsorbed

a = Calculated following Sakai et al. (2007)
b = stem weight calculated following Hosoda & Iehara (2010). Then, from stem weight, sapwood weight was calculated following Pâques et al. (2000).
c = Calculated following Hosoda & Iehara (2010).
* $P = 0.05$ ** P= 0.01

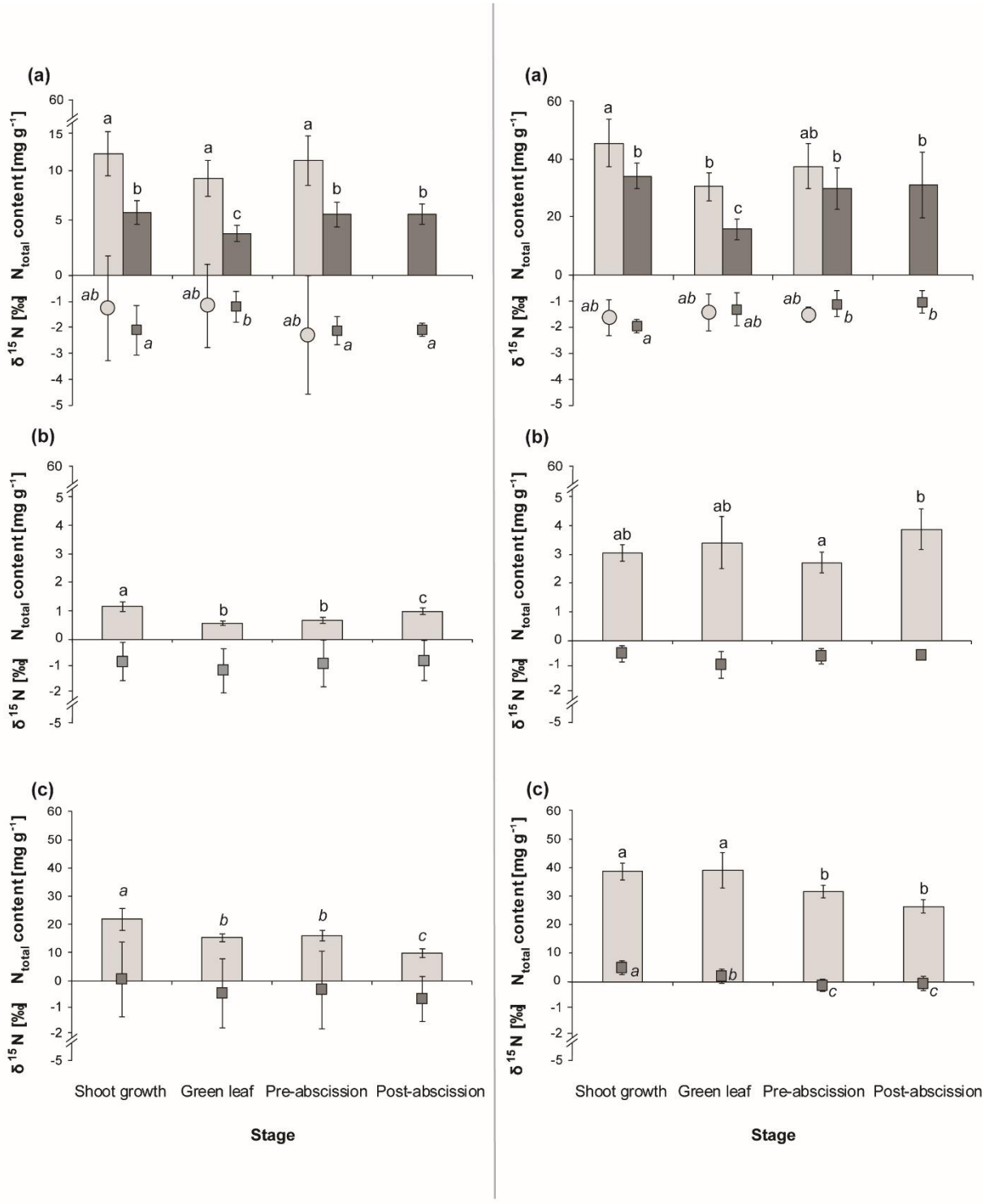


Figure 1 (*left*) and 2 (*right*): Seasonal pattern of N content and $\delta^{15}N$ in fine roots (light grey, circles) & coarse roots (dark grey, squares) **(a),** sapwood **(b)** and leaves **(c)** of *Larix Kaempferi* Lamb. (n=8) (Figure 1, left) and *Robinia pseudoacacia* L. (n=5) (Figure 2, right) The error bars denote SD, letters indicate differences in nitrogen content, *italic* letters indicate differences in isotopic composition (min. $P < 0.05$).

3.3 Amino acid analysis in plant tissues

For *Larix kaempferi* total free amino acid content (FAAC) in fine roots did not change throughout the phenological stages while single amino acids did change significantly (Fig. 3a). High concentrations of only alanine and glutamine were found, while other amino acids were found in minor concentrations most of which showed higher values during the shoot growth and pre-abscission stage and lower values during the green leaf stage (Appendix I). For *Robinia pseudoacacia*, fine roots showed the lowest ($P < 0.01$) FAAC during the green leaf period and was in general five to ten times higher than in *Larix kaempferi* depending on the phenological stage (Appendix II). Major constituents were proline followed by asparagine and alanine while the content of all measured amino acids generally was high in the shoot growth and pre-abscission stage and lowest in the green leaf stage (Figure 3b).

Coarse roots FAAC of *Larix kaempferi* did not show significant variation throughout the year. Major amounts of alanine, asparagine and glutamine were found, while other amino acids were found in minor concentrations generally being higher during the shoot growth stage and lower during the green leaf stage. Similarly, along the phenological stages, coarse roots FAAC of *Robinia pseudoacacia* did not vary significantly and was ten times higher than in *Larix kaempferi*. Like in fine roots, mainly proline, asparagine and alanine were found.

FAAC of the sapwood of *Larix kaempferi* did not vary in FAAC along the phenological stages and was made up mostly of arginine, glutamine and alanine, but the concentrations were extremely low. Similarly, sapwood FAAC of *Robinia pseudoacacia* did not vary along the phenological stages with contents ten times higher than the sapwood of *Larix kaempferi* and made up mostly of proline, asparagine and alanine.

Leaf FAAC of *Larix kaempferi* decreased ($P < 0.01$) from the shoot growth to the green leaf stage by 60% followed by a drastic increase of 430% during the pre-abscission stage. In the post-abscission stage, FAAC decreased by 65%. This final amount during the post-abscission stage was 50% of that found during the shoot growth stage. Throughout the phenological stages, alanine was the most abundant free amino acid with the highest content during the pre-abscission stage. Other free amino acids were found in low, varying concentrations throughout the year with generally higher amounts found in the pre-abscission and/or post-abscission stage. Leaves FAAC of *Robinia pseudoacacia* tended to decrease during the post-abscission stage and was mostly formed by Proline, alanine, asparagine, valine, isoleucine

and leucine with no clear pattern throughout the phenological stages except for alanine, which decreased significantly ($P < 0.05$). FAAC was two to ten times higher than in *Larix kaempferi*.

On average, for plant tissues of *Larix kaempferi* and *Robinia pseudoacacia*, FAAC was the highest in leaves and fine roots, followed by coarse roots and sapwood.

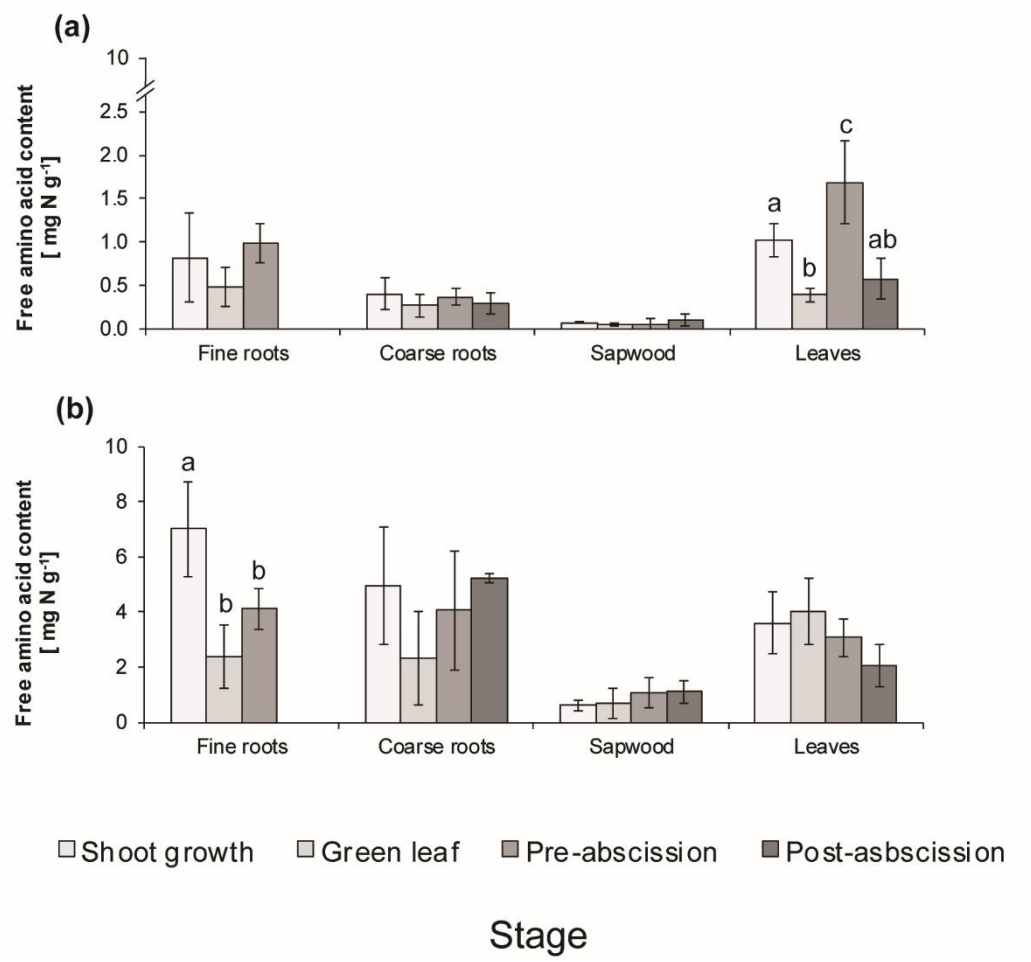


Figure 3: Seasonal pattern of total free amino acids content in fine roots, coarse roots, sapwood and leaves of **(a)** *Larix Kaempferi* Lamb. (n=8) and **(b)** *Robinia pseudoacacia* L. (n=5). The error bars denote SD, letters indicate differences in free amino acid content ($P < 0.01$).

4 Discussion

The N cycle in *Larix kaempferi* and *Robinia pseudoacacia* followed a well-established pattern: in the green leaf stage, N was bound in leaves photosynthetic apparatus (Ueda e al., 2011). During the pre-abscission stage, leaf N was reabsorbed and stored. Subsequently, in the post-abscission stage, leaves were abscised and became fresh litter that was depleted in N content (*NRE** 52% and *NRE** 47%, respectively). The value for *Larix kaempferi* was slightly lower than that found by Enta, et al. (2019) (*NRE** = 63%) in a stand nearby, possibly due to lower reabsorption of N found in their study as revealed by the *NRP* (0.8 %N) (Enta et al., 2019) vs 1.0 %N in our study). Further, they found for *Robinia pseudoacacia* a slightly lower value *NRE* (*NRE** 35%), which was in agreement with the lower *NRP* (3.2). Both differences could be linked to a difference in sampling time before leaf abscission and differences in annual climatic conditions, as the study by Enta et al. (2019) was conducted in the same area in 2016 which was significantly less snowy (February 2016: 2.5m, February 2017: 4.5m, data from the meteorological station at the site) affecting all forest processes due to a longer snow melting period. Further, during the shoot growth stage, stored N was remobilized and soil N uptaken to be transported upwards to support shoot growth in both species (Gessler et al., 1998).

N content and FAAC of all plant tissues was considerably higher in *Robinia pseudoacacia* because of its inherent characteristics of fixing high amounts of N from the atmosphere (Rice et al. 2004; Malcom et al., 2008; Lopez et al, 2014).

In both species, we found a temporal change in N isotopic composition between plant tissues, due to plant internal N transformations (Kolb & Evans, 2002; Millard & Grelet, 2010) with roots being the most depleted.

As hypothesized, we found a temporal pattern of N storage, first in the coarse roots in the pre-abscission stage followed by the sapwood in the post-abscission stage in both species. This is in agreement with findings for evergreen Japanese cedar and deciduous Japanese beech in this area (Seidel et. al., 2019a; b) indicating that this may be a general pattern for tree species of the cold temperate zone.

Further, whole tree measurements revealed, that both species predominantly stored N in coarse roots during the pre-abscission stage. Further, *Larix kaempferi* stored primarily soil

uptaken N, while *Robinia pseudoacacia* stored largely leaf reabsorbed N demonstrating its reliance on this internal N source.

4.1 *Shoot growth stage*

During this stage, FAAC and N content were high in all tissues for both species. N remobilization from storage tissues and uptake from soil sources started in *Larix kaempferi* while *Robinia pseudoacacia* up took N from the atmosphere and remobilized N from storage tissues (Millard & Grelet, 2010).

For *Larix kaempferi* the abundance of alanine in all tissues and arginine and glutamine in the woody tissues indicated their significant role during N transport to leaves where free amino acids are synthesised to proteins to support photosynthesis as found also for Japanese cedar and Japanese beech (Seidel et al., 2019a; b). Arginine and glutamine appeared to be the predominant amino acids for transport of N, as it has also been shown by Bi et al. (2004) and Seidel et al. (2019a) for arginine in young almond trees and Japanese beech respectively and for glutamine for Japanese cedar (Seidel et al. 2019b) and for cherry and poplar trees (Millard et al. 2006).

Growth of new leaves started already before the snow-melting season has concluded when N root uptake was still limited, demonstrating the importance of remobilization of stored N (Grassi et al., 2002; Millard et al., 2006). Grassi et al. (2002) found that glutamine mainly transported remobilized N, while asparagine and aspartic acid transported soil uptaken N. Their results are in agreement with our findings for *Larix kaempferi* where glutamine content was higher in sapwood and roots in the shoot growth stage and lower during the other phenological stages, indicating transport of remobilized N. Meanwhile, asparagine and aspartic acid tended to show increasing concentrations in roots from the shoot growth to the pre-abscission stage and stable values in the sapwood throughout the sampling period. This may indicate that as stored N content decreased and soil temperatures increased at the end of the tree dormancy period, soil N uptake and transport from the roots increased.

Proline, asparagine and alanine were the dominant free amino acids in all plant tissues of *Robinia pseudoacacia* with proline being the most abundant. Proline is usually abundant in organisms under biotic or abiotic stress (Verbruggen & Hermans, 2008). The high amounts of proline in leaves during the shoot growth stage could be related to temperature stress in the

analysed trees, which start growing in April when snow depth is still 2 m and soil temperature was close to 0 °C (data not published). During the winter months (min. temperatures of -8.4°C) and the snowmelt period (March to May), low temperature stress (Kai & Iba, 2014) possibly triggered the biosynthesis of proline in leaves in the shoot growth period. This was also found by Seidel at al. (2019a) for Japanese cedar. Once the temperature stress decreased, proline partly degraded or synthesised with other compounds into proteins as shown by the low proline content in the green leaf stage (Heuer, 1999; Verbruggen & Hermans, 2008). Similar to *Larix kaempferi*, asparagine and alanine seemed to transport N from the woody storage tissues to leaf tissues (Grassi et al., 2002; Seidel et al., 2019a; b). Moreover, fine roots FAAC was exceptionally high, indicating uptake of N from the soil from the available N and DON pool possibly influencing the tree species $\delta^{15}N$ composition (Jones et al., 2004; Finzi & Berthrong, 2005). This soil N uptake and interactions with possibly comparable mycorrhizal fungi communities in combination with plant internal N transformations (Kolb & Evans, 2002; Millard & Grelet, 2010) may cause the woody plant tissues overall $\delta^{15}N$ value to shift away from the atmospheric $\delta^{15}N$ value, closer towards soil $\delta^{15}N$ value causing plant tissues of *Robinia pseudoacacia* to be similar to *Larix kaempferi* plant tissues in their $\delta^{15}N$ value. This could be validated by mycorrhizal identification, as both species soil $\delta^{15}N$ value is similar and only fungi could cause a difference in coarse roots $\delta^{15}N$ value from the soil as a N source (Hobbie & Högberg, 2012). However, leaves $\delta^{15}N$ value of *Robinia pseudoacacia* would most likely not be influenced strongly by this, as they are constantly uptaking atmospheric N, dampening any effect of soil $\delta^{15}N$ influencing leaf tissues $\delta^{15}N$. Thus, similar mycorrhizal communities and/or plant internal N transformations in *Larix kaempferi* must be causing $\delta^{15}N$ values close to atmospheric $\delta^{15}N$.

4.2 *Green leaf stage*

During this stage, all tissues of *Larix kaempferi* showed reduced N content indicating that stored N in coarse roots and sapwood was mostly remobilized (Grassi et al., 2002; Millard et al., 2006), while leaf N content decreased due to an increase in biomass in comparison to the shoot growth period. There was a reduction of roots N content in *Robinia pseudoacacia* similar to the reduction observed for *Larix kaempferi* and since N was predominantly stored in roots and not in sapwood, sapwood content did not change during remobilization of

stored N. Leaves N content did not decrease possibly due to continuous uptake of atmospheric N (Malcom et al., 2008).

For both species, FAAC was low in all tissues in comparison to all other phenological stages as most of them are bound in leaves (Ueda et al., 2011) indicating that the need for N transport was lower, as N storage was depleted and only soil derived N needed to be passed on (Millard et al., 2006). The exception was for leaves of *Robinia pseudoacacia*, showing no change in FAAC which may be linked to the constant uptake of N from the atmosphere.

Other studies measuring N concentration in different tissues of *Larix* trees are scarce. The N content of all plant tissues in our site was lower than those observed for *Larix sibirica* in Mongolia during the green leaf stage (Hayashi et al., 2018), we discovered that N content of all plant tissues was lower in our samples especially in woody tissues (~50% lower). This may be linked to higher ammonium and nitrate content found in the Mongolian soils of that study, as well as to a stronger link between ectomycorrhizal fungi (ECM) and the tree roots (Bi et al., 2004; Millard & Grelet, 2010). Further, a higher litter N content in *Larix sibirica* may be explained by the harsh Mongolian winter temperatures (mean annual temperature (MAT) -3.6°C to 1.7°C, which are much lower than in our plots (MAT 9.7°C). To protect leaves from freezing, *Larix sibirica* might have thicker cell walls than the *Larix kaempferi* trees in our plots causing them to have a greater immobile leaf N content as suggested by González-Zurdo et al. (2015). They found, that evergreen tree species (*Pinus pinaster, Quercus suber, Quercus ilex ssp. Ballota*) along decreasing temperature gradients did not change in leaf N content. However, greater amounts of N were bound in the cell walls to thicken them as a protection against temperature stress causing litter to have a higher N content.

Further, due to the soil conditions, the ECM reliance of *Larix sibirica* and the differences in MAT, mean annual precipitation and elevation, a comparison of the isotopic composition of plant tissues of *Larix sibirica* and *Larix kaempferi* is difficult (Vergutz et al., 2012). Yet, the isotopic composition of litter and stem samples were similar (Hayashi et al., 2018) to the ones found in the present study, showing a strong control of the intra-plant N transformation processes on the isotopic composition in these plant tissues.

Additionally, we found fractionation of $\delta^{15}N$ in coarse roots of *Larix kaempferi* during the green leaf stage temporally coinciding with a decrease in N content, indicating that during N transport from roots to leaves and depletion of roots N storage, discrimination of ^{15}N

occurred. This could not be attributed to a single amino acid, as the SD of single free amino acids was high. However, the change in coarse roots $\delta^{15}N$ had no effect on sapwood or leaf $\delta^{15}N$ as both tissues already had a lighter $\delta^{15}N$ value being similar to coarse roots $\delta^{15}N$ during the green leaf stage.

N content and isotopic composition of tissues of *Robinia pseudoacacia* however, were in the typical range of this species (Rice et al. 2004; Malcom et al., 2008; Lopez et al, 2014; Enta et al. 2019), closer to the atmospheric $\delta^{15}N$ (0 ‰).

4.3 Pre-abscission stage

Our data showed that during the pre-abscission stage for *Larix kaempferi*, the content of free amino acids in leaves peaked, where 9 out of 19 measured amino acids increased and 7 tended to increase in content, which were released via protein hydrolysis (Warren et al., 2003) with alanine being the most abundant. This had no effect on leaf $\delta^{15}N$ or total N content of the leaves showing that N remained in the leaves in the form of free amino acids at the time of sampling. At the same time, coarse roots N content significantly increased showing that N was stored in this tissue possibly in the form of proteins synthesised from alanine and asparagine uptaken by fine roots which were significantly increased in alanine and asparagine content. Further, coarse roots $\delta^{15}N$ decreased back to shoot growth stage levels, most likely influenced by freshly soil uptaken N on this plant tissue. This was further supported by whole-tree measurements showing, that most stored N is made up of soil N. During this stage, we found no change in N or amino acids content in the sapwood. This dataset indicated clearly that during the pre-abscission period, N was not yet reabsorbed from leaves and thus, the storage of N observed in the coarse roots may be the result of soil N uptake.

In general, fine and coarse roots showed high FAAC which was likely derived from soil uptake. The soil soluble N pool was mostly made up of DON representing a major plant available N pool being largely composed of amino acids, which can be rapidly uptaken by roots (Jones et al., 2004). In this study, we found that 70% of soluble N consisted of DON, which is in the range found for other cambisols in the same research area (Seidel et al., 2019a), possibly influenced by vegetation and climate affecting microorganisms and the N

cycle (Michalzik & Matzner, 1999; Neff & Hooper, 2002), explaining the high FAAC found in fine and coarse roots in all phenological stages.

In comparison, at the time of sampling, *Robinia pseudoacacia* already had started leaf N reabsorption as indicated by reduced leaf N content and FAAC temporally coinciding with increased FAAC in the sapwood transporting N to the roots for N storage. Additionally, leaf $\delta^{15}N$ decreased while coarse root $\delta^{15}N$ increased, indicating that reabsorbed leaf N was stored in coarse roots. This was further supported by the results of whole-tree measurements suggesting that most stored N was composed of reabsorbed leaf N while this could not be attributed to a single free amino acid.

4.4 *Post-abscission stage*

Shortly before leaves were abscised, leaf N was reabsorbed, as litter N and amino acids decreased significantly in *Larix kaempferi* and slightly in *Robinia pseudoacacia*. This indicated that mobile forms of N have been partially removed and mainly structural N (e.g. N bound in cell walls) remained in the leaves (Warren & Adams, 2004; Ueda e al., 2011). No fractionation of ^{15}N took place in *Larix kaempferi* during reabsorption, which was in agreement with Kolb & Evans (2010). On the other hand, *Robinia pseudoacacia* did fractionate during leaf N reabsorption showing that fractionation with leaf N reabsorption is species depended (Seidel et al., 2019b).

As N was reabsorbed from leaves, N content in the sapwood significantly increased in both tree species, indicating that leaf reabsorbed N might be partially stored in this tissue. However, as there was no change in isotopic composition of any tissue during this phenological stage, we could not determine whether reabsorbed leaf N was stored only in coarse roots or also in sapwood. Further, the leaf N contribution to the overall tree N storage in *Larix kaempferi* was small (7%), suggesting that the remaining 93% of the stored N was most likely made up of N uptaken from the soil, thus dampening any possible change in isotopic composition that may be caused by storage of reabsorbed leaf N. In addition, FAAC could not help to elucidate this issue either. During the post-abscission stage, coarse roots stored 80% N making it the major N storage tissue for *Larix kaempferi*. Alanine, glutamine and arginine were the most abundant free amino acids in coarse roots and sapwood indicating their function as storage forms of N (Bi et al., 2005) as found for Japanese cedar

(Myashita et al., 2007; Seidel et al, 2019a) and Scots pine (Näsholm & Ericsson, 1990; Nordin et al. 2001). Further, asparagine content in coarse roots peaked during the pre-abscission stage but dramatically decreased in the post-abscission period, indicating, that it is not an N form for storage. This is in agreement with previous studies (Bi et al, 2004; Bencúr et al. 2005; Lea et al., 2007; Seidel et al., 2019b) reporting that the main function of asparagine is to transport N after which it will be synthesised with other compounds into proteins for storage of N.

FAAC of *Robinia pseudoacacia* was still predominantly made up of proline possibly triggered by temperature stress and alanine as a form of N storage as described above. Further, asparagine also remained in coarse roots and sapwood, contradicting its previously assumed function as a transport and not storage form of N in *Larix kaempferi* (Bi et al, 2004; Bencúr et al. 2005; Lea et al., 2007; Seidel et al., 2019b). The reason for this remained unclear but may be linked to the leguminous nature of this tree species as it exudates asparagine from the roots (Smith, 1969).

5 Conclusion

Based on the results obtained in this study, we concluded that the temporal storage pattern of N first occurs in the coarse roots followed by storage in the sapwood, which appears to be a general pattern for trees of the cold temperate zone with *Robinia pseudoacacia* reabsorbing leaf N earlier than *Larix kaempferi*. Future studies should investigate if this temporal pattern of N storage is also found in other areas and tree species to confirm our findings. Stable isotopic analysis in combination with free amino acid content and whole-tree measurements proved to be useful tools to efficiently trace movement of N in *Larix kaempferi* and *Robinia pseudoacacia*. These tools helped to further elucidate the reasons why $\delta^{15}N$ in plant tissues of both species was similar although their N sources differ by revealing soil N uptake and minor contribution to N storage in *Robinia pseudoacacia*. Additionally, our results hinted towards possible similarities in mycorrhizal communities' composition and plant internal processes causing similarities in $\delta^{15}N$ in plant tissues of these two tree species which needs to be addressed in future studies. *Larix kaempferi* showed no fractionation during leaf N reabsorption and no changes in free amino acid content of N storage tissues while *Robinia pseudoacacia* showed no significant changes of FAAC in leaves or N storage tissues. Thus, we were not able to link changes in single amino acids content to changes in $\delta^{15}N$.

Acknowledgements

We want to thank the staff of the University Research Forest of Yamagata University for keeping the site accessible in summer and winter. Further, we thank Cristina Lerda from the Università degli Studi di Torino, for the measurements related to soil N forms.

Author contributions

Felix Seidel and M. Larry Lopez C. have done the conceptualization and methodology for this study. Felix Seidel conducted the investigation, formal analysis, data curation & visualization and prepared the original draft. M. Larry Lopez C. was supervising this study. Akira Oikawa measured Amino acids content, while Toshiro Yamanaka measured N content and isotope ratio. M. Larry Lopez C., Akiro Oikawa, Eleonora Bonifacio and Luisella Celi contributed to data interpretation, writing, and reviewing of the manuscript.

Appendix I: Amino acid content in different tissues of *Larix kaempferi* Lamb. (n=3). Only amino acids found in significant amounts were reported with ± denoting SD. Significant differences among same plant tissues are highlighted in bold.

Tissue	Period	total free amino acid content [mg N kg^{-1}]	Alanine [mg N kg^{-1}]	Arginine m[g N kg^{-1}]	Asparagine [mg N kg^{-1}]	Aspartic acid [mg N kg^{-1}]	Glutamic acid [mg N kg^{-1}]	Glutamine [mg N kg^{-1}]	Isoleucine [mg N kg^{-1}]
Fine roots	shoot growth	812 ± 511	138 ± 88	42 ± 34	32 ± 25	26 ± 31	42 ± 35	219 ± 100	41 ± 16
	green leaf	484 ± 222	73 ± 34	17 ± 10	60 ± 0.22	16 ± 9	52 ± 37	78 ± 58	25 ± 19
	pre-abscission	989 ± 221	**214 ± 26***	71 ± 56	**104 ± 89***	22 ± 7	92 ± 14	155 ± 85	32 ± 4
Coarse roots	shoot growth	404 ± 188	153 ± 83	59 ± 70	48 ± 38	14 ± 10	27 ± 23	145 ± 100	20 ± 9
	green leaf	**267 ± 133***	74 ± 4	13 ± 3	76 ± 111	7 ± 6	12 ± 4	15 ± 7	**6 ± 2***
	pre-abscission	366 ± 96	83 ± 15	32 ± 25	96 ± 138	8 ± 4	13 ± 2	17 ± 8	11 ± 4
	post abscission	**296 ± 122****	85 ± 24	37 ± 0.23	6 ± 1	5 ± 4	34 ± 18	39 ± 38	**9 ± 1***
Sapwood	shoot growth	70 ± 9	7 ± 3	1 ± 0	2 ± 1	2 ± 0	3 ± 2	22 ± 4	3 ± 0
	green leaf	46 ± 16	8 ± 5	5 ± 2	1 ± 0	2 ± 2	2 ± 1	12 ± 1	2 ± 0
	pre-abscission	55 ± 13	6 ± 6	7 ± 4	1 ± 0	2 ± 2	1 ± 1	**9 ± 4***	2 ± 2
	post abscission	101 ± 30	9 ± 3	4 ± 1	2 ± 2	2 ± 1	4 ± 1	**5 ±2***	3 ± 1
Leaf	shoot growth	1020 ± 187	183 ± 56	15 ± 6	28 ± 18	6 ± 2	14 ± 7	25 ± 16	68 ± 22
	green leaf	**392 ±79***	**116 ± 13***	9 ± 5	**4 ± 4***	**2 ± 1***	10 ± 3	23 ± 4	**4 ± 6***
	pre-abscission	**1687 ± 479***	**315 ± 30****	17 ± 9	33 ± 6	4 ± 1	21 ± 12	**72± 24****	**205 ± 21****
Litter	post abscission	**573 ± 234****	**46 ± 9****	11 ± 5	11 ± 6	4 ± 1	**39 ± 12****	**80 ± 24****	48 ± 21

*P = 0.05 **P =0.01

Appendix I (*continued*): Amino acid content in different tissues of *Larix kaempferi* Lamb. (n=3). Only amino acids found in significant amounts were reported with ± denoting SD. Significant differences among same plant tissues are highlighted in bold.

Tissue	Period	Leucine [mg N kg^{-1}]	Phenylanine [mg N kg^{-1}]	Proline [mg N kg^{-1}]	Serine [mg N kg^{-1}]	Threonine [mg N kg^{-1}]	Tryptophane [mg N kg^{-1}]	Tyrosine [mg N kg^{-1}]	Valine [mg N kg^{-1}]
Fine roots	shoot growth	36 ± 12	21 ± 8	40 ± 14	18 ± 9	27 ± 8	45 ± 56	11 ± 5	65 ± 25
	green leaf	**17 ± 9***	16 ± 8	**11 ± 3***	23 ± 16	21 ± 11	12 ± 0.1	15 ± 7	40 ± 28
	pre-abscission	40 ± 7	29 ± 9	**19 ± 6***	36 ± 10	56 ± 39	37 ± 0.37	18 ± 1	45 ± 4
Coarse roots	shoot growth	19 ± 10	18 ± 4	57 ± 35	15 ± 6	14 ± 5	45 ± 40	10 ± 4	35 ± 14
	green leaf	**6 ± 3***	**6 ± 1****	**10 ± 3***	11 ± 3	7 ± 2	2 ± 1	5 ± 1	**13 ± 5***
	pre-abscission	14 ± 3	**10 ± 3***	**18 ± 11***	14 ± 6	12 ± 5	10 ± 4	6 ± 3	18 ± 6
	post abscission	10 ± 4	**7 ±2****	**12 ± 4***	12 ± 2	8 ± 2	10 ± 7	**4± 2***	**14 ± 2***
Sapwood	shoot growth	3 ± 0	1 ± 1	8 ± 7	6 ± 3	2 ± 1	2 ± 0	2 ± 0	5 ± 1
	green leaf	2 ± 1	1 ± 0	1 ± 1	4 ± 2	1 ± 0	**0 ± 0***	1 ± 0	3 ± 1
	pre-abscission	2 ± 2	1 ± 1	2 ± 2	3 ± 3	1 ± 1	**0 ± 0***	1 ± 0	3 ± 3
	post abscission	3 ± 2	2 ± 1	4 ± 2	5 ± 1	2 ± 1	2 ± 1	2 ± 1	4 ± 2
Leaf	shoot growth	7 ± 1	79 ± 28	285 ± 11	40 ± 17	23 ± 14	23 ± 8	31 ± 11	112 ± 32
	green leaf	**2 ± 1***	**25 ± 10***	**129 ± 24****	**10 ± 10***	11 ± 11	11 ± 10	8 ± 4	**19 ± 10***
	pre-abscission	**23 ± 5****	119 ± 11	**101 ± 18****	**73 ± 7***	116 ± 8	**38 ± 9****	**22 ± 3***	**216 ± 22****
Litter	post abscission	7 ± 6	**19 ± 11***	**29 ± 18****	**20 ±7***	24 ± 8	21 ± 9	8.00 ± 3	**57 ± 22***

*P = 0.05 **P =0.01

Appendix II: Amino acid content in different tissues of *Robinia pseudoacacia* L. (n=3). Only amino acids found in significant amounts were reported with ± denoting SD. Significant differences among same plant tissues are highlighted in bold.

Tissue	Period	total free amino acid content [mg N kg^{-1}]	Alanine [mg N kg^{-1}]	Arginine m[g N kg^{-1}]	Asparagine [mg N kg^{-1}]	Aspartic acid [mg N kg^{-1}]	Glutamic acid [mg N kg^{-1}]	Glutamine [mg N kg^{-1}]	Isoleucine [mg N kg^{-1}]
Fine roots	shoot growth	7008 ± 1706	526 ± 189	1334 ± 52	1334 ± 655	277 ± 42	270 ± 31	146 ± 10	339 ± 45
	green leaf	**2372 ± 1166****	**194 ± 56***	**840 ± 10***	840 ± 779	**151 ± 69***	**109 ± 29****	**74 ± 37***	**72 ± 29****
	pre-abscission	4121 ± 748	367 ± 23	944 ± 22	945 ± 437	178 ± 17	**214 ± 35***	**68 ± 22***	**68 ± 14****
Coarse roots	shoot growth	4950 ± 2124	333 ± 106	144 ± 137	1458 ± 695	133 ± 54	90 ± 47	67 ± 12	171 ± 81
	green leaf	2325 ± 1702	**141 ± 68***	23 ± 34	909 ± 111	87 ± 45	44 ± 9	49 ± 52	**47 ± 37***
	pre-abscission	4052 ± 2166	301 ± 77	152 ± 221	1498 ± 142	122 ± 34	84 ± 27	63 ± 40	**36 ± 5***
	post abscission	5228 ± 169	339 ± 52	135 ± 118	1290 ± 134	132 ± 39	64 ± 16	63 ± 24	53 ± 25
Sapwood	shoot growth	610 ± 177	85 ± 35	16 ± 12	180 ± 83	18 ± 6	8 ± 1	11 ± 5	26 ± 8
	green leaf	679 ± 531	127 ± 102	11 ± 15	279 ± 212	67 ± 56	14 ± 5	26 ± 24	16 ± 11
	pre-abscission	1075 ± 565	219 ± 101	5 ± 5	261 ± 186	76 ± 62	40 ± 31	39 ± 32	11 ± 4
	post abscission	1097 ± 421	150 ± 61	11 ± 10	180 ± 116	31 ± 18	26 ± 20	19 ± 18	19 ± 12
Leaf	shoot growth	3600 ± 1108	802 ± 126	21 ± 7	195 ± 130	89 ± 15	55 ± 7	25 ± 3	157 ± 22
	green leaf	4029 ± 1188	893 ± 113	28 ± 7	**556 ± 88***	198 ± 171	50 ± 33	51 ± 40	330 ± 136
	pre-abscission	3068 ± 670	1052 ± 389	6 ± 2	170 ± 47	**23 ± 7***	65 ± 11	39 ± 26	140 ± 73
Litter	post abscission	2028 ± 764	**196 ± 60***	14 ± 3	285 ± 177	**9 ± 4***	58 ± 8	79 ± 16	215 ± 75

**P* = 0.05 ***P* =0.01

Appendix II (*continued*): Amino acid content in different tissues of *Robinia pseudoacacia* L. (n=3). Only amino acids found in significant amounts were reported with ± denoting SD. Significant differences among same plant tissues are highlighted in bold.

Tissue	Period	Leucine [mg N kg^{-1}]	Phenylanine [mg N kg^{-1}]	Proline [mg N kg^{-1}]	Serine [mg N kg^{-1}]	Threonine [mg N kg^{-1}]	Tryptophane [mg N kg^{-1}]	Tyrosine [mg N kg^{-1}]	Valine [mg N kg^{-1}]
Fine roots	shoot growth	218 ± 22	208 ± 90	2050 ± 505	233 ± 43	223 ± 70	133 ± 48	90 ± 30	526 ± 91
	green leaf	**52 ± 22***	**64 ± 19***	**396 ± 154****	**52 ± 25****	**68 ± 31***	82 ± 17	**34 ± 26***	**132 ± 55****
	pre-abscission	**52 ± 5***	**60 ± 5***	**1555 ± 170****	**69 ± 6****	**94 ± 10***	92 ± 35	**25 ± 1***	**91 ± 12****
Coarse roots	shoot growth	101 ± 36	130 ± 60	1238 ± 363	142 ± 71	106 ± 54	79 ± 33	57 ± 28	309 ± 186
	green leaf	**30 ± 26***	**35 ± 26***	**596 ± 102***	**40 ± 18***	84 ± 75	93 ± 42	24 ± 18	**71 ± 29***
	pre-abscission	**34 ± 6***	**41 ± 2***	1263 ± 407	53 ± 18	66 ± 27	80 ± 114	23 ± 15	**61 ± 10***
	post abscission	**32 ± 10***	69 ± 25	**2527 ± 198****	79 ± 29	73 ± 38	27 ± 4	22 ± 11	111 ± 50
Sapwood	shoot growth	16 ± 4	8 ± 1	134 ± 22	20 ± 8	12 ± 4	10 ± 2	8 ± 2	45 ± 17
	green leaf	8 ± 6	**2 ± 0***	**31 ± 6***	25 ± 24	12 ± 8	**18 ± 2***	7 ± 7	23 ± 20
	pre-abscission	9 ± 3	5 ± 1	**320 ± 98***	28 ± 16	15 ± 10	7 ± 5	3 ± 2	22 ± 13
	post abscission	12 ± 7	11 ± 6	**543 ± 137****	24 ± 11	12 ± 6	5 ± 4	3 ± 1	36 ± 22
Leaf	shoot growth	128 ± 25	76 ± 12	1227 ± 565	234 ± 53	100 ± 20	81 ± 31	84 ± 15	257 ± 48
	green leaf	170 ± 23	78 ± 9	556 ± 197	**126 ± 11***	219 ± 23	118 ± 108	103 ± 56	**461 ± 52***
	pre-abscission	108 ± 52	49 ± 28	837 ± 132	**139 ± 33***	73 ± 27	67 ± 19	23 ± 9	220 ± 114
Litter	post abscission	160 ± 60	**121 ± 43***	**364 ± 181***	**82 ± 31****	71 ± 20	38 ± 29	**9 ± 1***	282 ± 112

*P = 0.05 **P =0.01

Chapter 5

Seasonal nitrogen and phosphorous cycling in four Japanese cool-temperate forest species

Felix Seidel[1], M. Larry Lopez C.[1], Luisella Celi[2], Eleonora Bonifacio[2], Hiroko Kurokawa[3]

[1]Faculty of Agriculture, Yamagata University, Tsuruoka, Japan

[2]Department of Agricultural, Forest and Food Sciences, Università degli Studi di Torino, Torino, Italy

[3]Forestry and Forest Products Research Institute, Tsukuba, Japan

Author for correspondence:

Felix Seidel

Tel.: +81-235-28-2961

E-Mail: seidel.geo-science@gmx.de

Faculty of Agriculture Yamagata University

Tsuruoka-shi, Wakabamachi 1-23, 997-8555

Yamagata Prefecture, Japan

Abstract

Background and Objectives: In soil-plant systems the P dynamics during the whole annual cycle in leaves, stem and root tissues, the allocation of P in the different tissues and the significance of P availability in soil for P allocation remain largely unknown, especially as related to N dynamics. Thus, our objectives were (1) to evaluate P reabsorption efficiency and proficiency among different tree species, (2) to correlate plant tissues N content with P content, and (3) to identify P storage tissues and their respective contribution to whole-tree P storage.

Materials and Methods: Three trees of four tree species were selected and coarse roots, sapwood, leaves, litter and soil samples were collected. Plant tissues were sampled during four phenological stages. Total and available N and P contents were measured and upscaled to whole-tree level.

Results: All species reabsorbed N and P in significant amounts. *Cryptomeria japonica* was most nutrient proficient and stored the largest amounts of N and P, while *Larix kaempferi* was highly N and P proficient but focused more on conserving P than N. *Fagus crenata* was highly efficient in N and P resorption but least proficient conserving N and P. *Robinia pseudoacacia* exhibited generally poor N and P reabsorption but was more efficient conserving P than N.

Conclusions: This study showed different tree species patterns of P resorption and storage, with evergreen *Cryptomeria japonica* storing large amounts of N and P making this species more resilient towards changes in soil N and P availability. Deciduous *Larix kaempferi* was low N and P demanding and able to grow well in nutrient poor soils. In contrast, broad-leaved *Fagus crenata* was highly N and P demanding and most efficient in absorbing N and P. Finally, leguminous *Robinia pseudoacacia* demonstrated clearly the effect of soil N independence on plant tissues P content, enabling it to acquire the highest amounts of N and P. The results of this study showed the particular interaction of N and P inherent to each tree species and shed new lights on different tree species nutrient budgets for forest management practices.

Keywords: *Cryptomeria japonica*, *Fagus crenata*, *Larix kaempferi*, *Robinia pseudoacacia, N and P dynamics, N:P ratio*

1 Introduction

The availability of nitrogen (N) and phosphorous (P) in ecosystems is a driving force of plant community composition as N and P are essential macronutrients supporting plant growth and development (Aerts & Chapin, 2000; Kolb & Evans, 2002; Güsewell & Gessner, 2009). Their limitation or imbalance in forest ecosystems can reduce plant growth and photosynthetic activity (Güsewell & Gessner, 2009; Zhang et al., 2014; Luo et al., 2015). N is vital for the formation of proteins, nucleic acids and chlorophylls (Warren & Adams, 2004; Ueda et al., 2011) affecting photosynthetic processes (Güsewell, 2004), while P is a vital component of RNA, DNA, phospholipids and sugar phosphates and involved in cellular energy transfer (Marschner, 1995; Rennenberg & Herschbach, 2013; Li et al., 2017). Based on soil P budget and availability, plants may develop highly efficient strategies for P uptake and utilization, P storage and mobilization and high resorption efficiencies (Rennenberg & Herschbach, 2013), leading to P cycling through the ecosystem.

However, there is only very limited information available regarding plant P cycling (Rennenberg & Herschbach, 2013; Li et al, 2017), while plant N cycling during the growing season has been extensively studied (Millard & Grelet, 2010). Further, many studies have been conducted on N and P fertilization in laboratory experiments in N-limited or P-limited conditions often with saplings (De Groot et al., 2003; Wright et al., 2005; Wright et al., 2011; Yavitt et al., 2011; Santiago et al., 2012; Mori et al., 2013), but studies with adult trees in forest ecosystems are scarce (Li et al., 2017). Information on P dynamics during the whole annual cycle in leaves, stem and root tissues, the contribution of those plant tissues to P storage, P mobilisation processes, and the significance of soil P availability in the soil on P plant allocation have so far not been shown (Rennenberg & Herschbach, 2013; Li et al., 2017).

Therefore, the present study aims to elucidate P cycling of four tree species to explore the seasonal changes of P, as related to N dynamics. This work builds up on the results described in Seidel et al. (2019a, b, c) presenting comprehensively the N cycling of *Cryptomeria japonica*, *Fagus crenata*, *Larix kaempferi* and *Robinia pseudoacacia*. These tree species are of four different functional groups: evergreen conifer (*Cryptomeria japonica*), deciduous conifer (*Larix kaempferi*), deciduous broad-leaved (*Fagus crenata*), and deciduous leguminous (*Robinia pseudoacacia*) exhibiting different nutrient demands. *Larix kaempferi*

took up very low amounts of N, followed by *Cryptomeria japonica*, while *Fagus crenata* absorbed significantly more N. *Robinia pseudoacacia* took up the largest N amount (Seidel et al. 2019a, b, c). Li et al. (2017) stated, that a higher N content leads to an increased P demand as higher N content enables plants to increase their leaf area index, extend photosynthesis duration and improve nutrient uptake with all of these processes requiring P. This was supported by Kang et al. (2010), presenting a significant positive correlation between N and P content. Thus, based on the N cycling of the four tree species (Seidel et al. 2019a, b, c), we aim to clarify the P cycle by means of seasonal measurements of coarse roots, sapwood and leaves.

We hypothesized that all four tree species belonging to different functional groups will reabsorb leaf P that is subsequently stored in sapwood and roots after leaf abscission and further, that these tree species will exhibit different P storage strategies depending on their species and soil P availability. Our objectives were (1) to evaluate P reabsorption efficiency and proficiency among species, (2) to correlate plant tissue P to N content and soil P availability, and (3) to identify P storage tissues and their respective contribution to whole-tree P storage.

2 Materials and Methods

2.1 Study site

The research site is located in north-eastern Japan in the Research Forest of Yamagata University on the Japanese Sea side. The humid climate is characterized by an annual precipitation of 3000 mm, with approximately half falling in the form of snow, covering the sampling sites from December to May at an elevation of 300 to 700 m above sea level (Fig. 1). The sampling sites distributed within a radius of 3 km and were selected by accessibility, stand age and stand purity. Three Japanese cedar trees (*Cryptomeria japonica* D. Don.) were sampled in a 50-year old plantation composed of exclusively *Cryptomeria japonica* with an understory vegetation of broad-leaf bamboo (*Sasa veitchii*) growing at an elevation of ~300m above sea level. Three 40-50-year old Japanese larch trees (*Larix kaempferi* Lamb.) in a mixed forest dominated by *Larix kaempferi* (~80-90%) were chosen, as there were no pure stands in the Yamagata University Research Forest. Finally, three 70-80 years old Japanese

beech trees (*Fagus crenata* Blume) growing in a pure stand at the highest elevation (~700 m above sea level) were selected. Three 7-year old Black locust trees (*Robinia pseudoacacia*) growing in a pure stand were sampled. Prior to the colonization of the area by Black locust, a *Cryptomeria japonica* plantation was present, which was harvested and subsequently burned. After the fire, *Robinia pseudoacacia* quickly invaded the plot (Table 1).

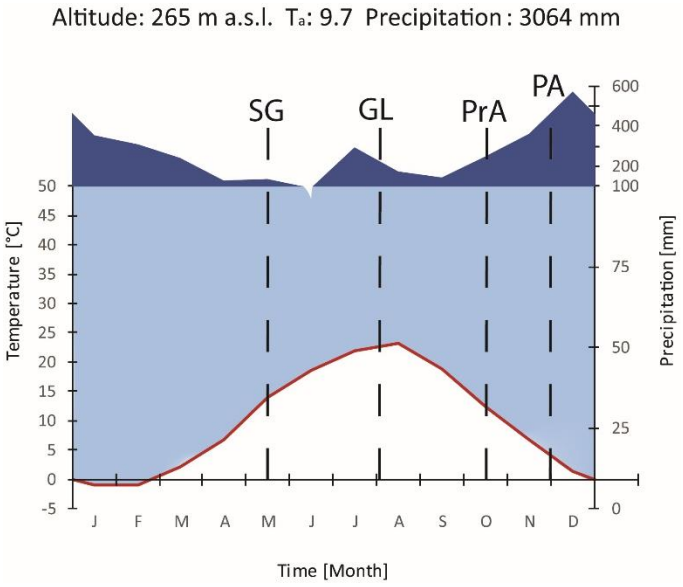


Figure 1: Meteorological conditions at the Yamagata University Research Forest. Dashed lines indicate sampling times: SG = shoot growth period, GL = green leaf period, PrA = pre-abscission period, PA = post-abscission period (from Seidel et al., 2019a).

Table 1: Characteristics of sampled tree species (n = 9).

Site	Coordinates	m a.s.l.	Tree	DBH	Height	Age
Cryptomeria japonica	N38° 32.987' E139° 51.801'	295	A	41.3	21.1	54
			B	39.9	27.7	54
			C	44.6	22.5	54
Larix kaempferi	N38° 33.427' E139° 52.560'	650	A	42.3	25.9	48
			B	35.0	21.1	45
			C	35.6	24.8	47
Fagus crenata	N38° 33.390 E139° 52.630'	690	A	30.7	25.4	71
			B	30.2	24.4	71
			C	29.9	43.2	73
Robinia pseudoacacia	N38° 33.560' E139° 52.125'	405	A	10.7	10.1	7
			B	10.9	12.5	7
			C	13.2	12.9	7

DBH= diameter at breast height

2.2 Plant and soil sample collection and treatments

Coarse roots (> 2 mm), sapwood and leaf samples were collected from May 16 to 18 (shoot growth stage), August 1 to 3 (green leaf stage), October 19 (pre-abscission stage), and on November 29 (post-abscission stage) with fresh litter collected from litter traps (size: 1 m², placed on May 15 for *Cryptomeria japonica* and on September 4 for the other species). Coarse roots of single trees were dug out, identified and cut from the trunk up to a depth of 30 cm. Three samples surrounding each tree were taken and pooled. Whole tree cores were collected by using an increment borer with a 10 mm diameter for *Larix kaempferi* and *Cryptomeria japonica* while a 5 mm borer was chosen for the hard woods *Fagus crenata* and *Robinia pseudoacacia.* Sapwood and heartwood were identified and separated by colour. Heartwood was sampled only once, assuming that there was no variation in P content throughout the growing season as P moves with water in the sapwood (Rennenberg & Herschbach, 2013). Three samples of heartwood were taken in the green leaf period of 2017 on August 3. Leaves without insect attacks were sampled from the middle of the canopy to avoid effects of crown position and by sampling different leaf clusters, we included possible variations in P content (Rosecrance et al., 1998; Özbucak et al., 2008). Further, leaves of evergreen *Cryptomeria japonica* were separated and analysed by year, which has been described in more detail in Seidel et al. (2019a). All plant samples were transported in plastic bags and directly oven dried in the laboratory. All plant material was dried to a constant weight at 60°C for at least 48 hrs, following Rosecrance et al. (1998). Subsequently, they were ground and stored in a dark and cool place until further analysis.

Soil horizons were identified in the field following the FAO guidelines (FAO, 2006) and genetic horizons were sampled for soil characterization as described in Seidel et al. (2019a, b, c).

The humus layer was removed and only mineral soil samples were taken from three depths (0-5cm, 5-15cm and 15-30cm) from August 1 to 3, 2017. Within 30 cm distance around each tree, three small pits were sampled. All samples were taken from the field in a cooler box and air dried for at least 48 h and then sieved (<2 mm). Subsequently, the samples from the three small pits around each individual tree were pooled according to their depth, ground and stored in a freezer until further analysis.

2.3 Total and available P analysis

For plant and soil materials, total phosphorous (TP) was determined by digesting the samples with potassium peroxydisulfate. All samples were weighed and burned at 550°C for 2 hours. Potassium peroxydisulfate was added and samples positioned in an autoclave (LSX-300 High pressure steam sterilizer, TOMY, Japan) for 1 hour at 121°C and then centrifuged (3000 rpm, 5min). The concentration of TP was determined colorimetrically with a microplate reader (Multiscan GO, Thermo Scientific, USA).

Available P was determined on soil samples using both Bray and Olsen methods (Olsen et al., 1954).

2.4 Whole tree measurements

In August 2018, whole-tree harvest of three *Cryptomeria japonica* trees and three *Robinia pseudoacacia* trees was conducted to destructively collect biomass data of leaves and sapwood. Before cutting the trees, branches were cut off to reduce leaf loss through the falling stem. In the case of *Cryptomeria japonica*, three branches of four typical sizes (120-150 cm, 150-160 cm, 200-230 cm and 300-360 cm, respectively) were selected and leaves were removed and separated by age. For both species, leaves were dried at 50°C before weighing to estimate whole-tree leaf (separated by years for *Cryptomeria japonica*) weight. Every two meters a tree disc was cut from stems and dried at 50°C. They were separated into heart- and sapwood sections and based on the disk characteristics the volume and weight of the sapwood was calculated for these two species. For the coarse root weight, power equations by Lim et al. (2013) for *Cryptomeria japonica* and Blujdea et al. (2012) for *Robinia pseudoacacia* were used. For *Fagus crenata* and *Larix kaempferi*, leaf, sapwood and coarse roots weigh was estimated by power equations (Gebauer et al., 2008; Tateishi et al., 2010; Ono et al., 2013 and Pâques, 2001; Sakai et al., 2007; Hosoda & Iehara, 2010, respectively), which are described in detail by Seidel et al. (2019b,c). Tissue weight was calculated for the green leaf period and a change in tissue weight due to growth was assumed negligible from the green leaf stage until the post-abscission stage. We calculated changes in P content for all phenological stages except for the shoot growth stage, as we could not estimate whole-tree leaf weight during that time.

Furthermore, we assumed that in the post-abscission stage all P was in the expected storage tissues (coarse roots and sapwood) while storage was considered empty during the green leaf stage as P is needed in the growing tissues (Rennenberg & Herschbach, 2013). Thus, the whole-tree P storage was calculated as follows:

$$P_{storage} = P_{post} - P_{green}$$

Where $P_{storage}$ is the P stored in a certain tissue, P_{post} is the P content during the post-abscission stage and P_{green} is the P content during green leaf stage. We calculated this value for all plant tissues as whole-tree absolute P storage (g P).

Finally, reabsorption efficiency (*PRE*) was calculated as:

$$PRE = \left(1 - \frac{mass\ of\ P\ in\ senesced\ leaves}{mass\ of\ P\ in\ greeen\ leaves}\right) \times 100$$

P was expressed as mass per leaf dry mass (mg g^{-1}). Litter represents the senesced leaves in November, while green leaf is the matured leaf in August. We calculated whole-tree total reabsorbed leaf P by considering the total tree leaf weight scaled up from litter trap measurements. Further, we calculated PRE* to account for leaf mass loss during P reabsorption (Correction Factor (CF) for conifers 0.754) following Vergutz et al. (2012), which could lead to an underestimation of 10% (van Heerwaarden et al., 2003).

$$PRE^{*} = \left(1 - \frac{mass\ of\ P\ in\ senesced\ leaves}{mass\ of\ P\ in\ greeen\ leaves} * CF\right) \times 100$$

Subsequently, P resorption proficiency (PRP) was calculated, since it represents a more stable indicator of plant ability to recycle nutrients than PRE. Total P content per leaf mass in senescing leaves was used as PRP (Killingbeck, 1996; Richardson et al. 2005). Leaves that can reduce P concentration in senescing leaves to a lower level are more proficient in resorbing P. The PRP is expressed in (%) dry mass.

Finally, reabsorbed leaf P on whole-tree level was calculated as follows:

$$P_{whole-tree\ reabsorbed} = \frac{(L_P * L_{mass})}{100 - PRE} * PRE$$

$P_{whole\text{-}tree\ reabsorbed}$ was expressed as [g P], L_P was P content of litter [g/kg] and L_{mass} was the litter weight [kg] representing the whole tree loss of P [g P] with litter and finally PRE [%] as the P resorption efficiency. With the $P_{whole\text{-}tree\ reabsorbed}$ value we could calculate leaf P reabsorption contribution to whole-tree P storage [%].

2.5 Statistical analysis

One-way ANOVA was applied to determine the statistical significance of differences in P and available P content of all measured plant tissues and soil samples. If significant differences were found, post-hoc multiple comparison was conducted, using a Tukey-Kramer test at the significance levels of 0.05 and 0.01. Furthermore, simple linear regression analysis was conducted with RStudio (Version 1.1.463 – © 2009-2018 RStudio, Inc.) in order to evaluate the effect of the dependent variable (P content) on the independent variable (N content).

3 Results

3.1 Soil characteristics

The soils in all sampled plots were identified as brown forest soils (Cambisol) and P content did not change significantly with depth in the top 30 cm (Table 2). Available P decreased in all plots from top to bottom ($P < 0.05$) by at least 60%.

The results of our study showed that *Cryptomeria japonica* and *Robinia pseudoacacia* grew in plots with moderate total and available P content, *Larix kaempferi* grew on plots with the highest total P content but the lowest P availability ($P < 0.05$), *Fagus crenata* grew on soils with the lowest total P content but the highest available P content.

3.2 P content and reabsorption in plant tissues

All species showed no significant changes in coarse roots P content throughout the season (Fig. 2). The coarse roots of *Robinia pseudoacacia* showed the highest P content followed by *Cryptomeria japonica* ($P < 0.01$) having 30% lower P content. The coarse roots of *Larix kaempferi* and *Fagus crenata* had the lowest values ($P < 0.01$) being 50% lower than *Cryptomeria japonica*.

There was no significant change in sapwood P content throughout the seasons for *Larix kaempferi* and *Cryptomeria japonica* (Fig. 2). *Fagus crenata* sapwood P content decreased

Table 2: Mean ±SD of soil parameters under *Cryptomeria japonica*, *Larix kaempferi*, *Fagus crenata* and *Robinia pseudoacacia*. Significant differences among same parameters are highlighted in bold.

Species	Depth [cm]	total P [mg kg^{-1}]	available P [mg kg^{-1}]	total P : available P ratio	total N [mg kg^{-1}]	available N [mg kg^{-1}]	total N : available N ratio	Ammonium [mg N kg^{-1}]	Nitrate [mg N kg^{-1}]	DON [mg N kg^{-1}]	C : N
Cryptomeria japonica	0-5	1988 ± 1106	41.3 ± 20.9	48	9236 ± 3944	390 ± 111	24	137 ± 113	20 ± 21	230 ± 88	14 ± 4
	5-15	3356 ± 1677	21.5 ± 16.3	156	**5172 ± 1360****	**208 ± 42****	25	**34.8 ± 41****	**10 ± 12****	163 ± 56	14 ± 3
	15-30	2376 ± 984	**14.4 ± 11.9***	165	**3706 ± 834****	**132 ± 18****	28	**4.8 ± < 1****	**8 ± 8****	**120 ± 10****	14 ± 2
Larix kaempferi	0-5	3532 ± 2231	16.0 ± 8.5	221	3502 ± 2644	138 ± 58	25	8 ± 3	1 ± 1	129 ± 49	19 ± 6
	5-15	4658 ± 1459	10.0 ± 5.0	466	1782 ± 908	88 ± 8	20	5 ± 1	1 ± < 1	82 ± 8	18 ± 3
	15-30	3313 ± 2386	**6.7 ± 1.5***	494	**1438 ± 436***	**78 ± 6***	18	5 ± 1	1 ± < 1	79 ± 15	14 ± 6
Fagus crenata	0-5	937 ± 311	35.8 ± 16.0	26	7795 ± 2105	293 ± 91	26	253 ± 54	4 ± 6	39 ± 38	20 ± 2
	5-15	1859 ± 979	**20.8 ± 8.6***	89	**3591 ± 906****	**173 ± 36****	21	**100 ± 74***	3 ± 5	70 ± 81	20 ± 3
	15-30	1473 ± 631	**12.6 ± 4.0****	117	**1044 ± 214****	**141 ± 51****	7	**104 ± 77***	3 ± 4	33 ± 35	16 ± 3
Robinia pseudoacacia	0-5	1794 ± 697	34.0 ± 5.2	53	6063 ± 2290	143 ± 1	42	12 ± < 1	48 ± 6	83 ± 5	20 ± 2
	5-15	3320 ± 1465	**12.6 ± 6.0****	263	**2877 ± 1600***	**100 ± < 1****	29	5 ± < 1	18 ± < 1	77 ± 7	**12 ± 3***
	15-30	2417 ± 1530	**9.6 ± 3.2****	252	**1982 ± 576****	**76 ± < 1****	26	3 ± < 1	11 ± <1	58 ± 9	**11 ± 2****

*** *P* < 0.05** **** *P* < 0.01**

Total N, available N, total N : available N ratio, ammonium, nitrate, dissolved organic nitrogen (DON) and C:N values are taken from Seidel et al. (2019a,b,c)

(*P* < 0.01) from the shoot growth to the green leaf period by 55% and remained low the rest of the year. Sapwood of *Robinia pseudoacacia* showed an increase of 40% in P content from the green leaf period to the post-abscission period (*P* < 0.01). On average, *Robinia pseudoacacia* had the highest amount of P in the sapwood, followed by *Cryptomeria japonica* and *Fagus crenata* having 30% and 60% lower P content respectively (*P* < 0.01). *Larix kaempferi* had the lowest (*P* < 0.01) with a 70% lower P content in comparison with *Robinia pseudoacacia*. Heartwood P content was lower (*P* < 0.05) in *Larix kaempferi* and *Robinia pseudoacacia* in comparison to their respective sapwood P content during all phenological stages (Table 3). For *Fagus crenata* heartwood P content was significantly higher than the sapwood during the shoot growth stage while it was similar to the sapwood P content during all other phenological stages. There were no differences between heartwood and sapwood found for *Cryptomeria japonica*.

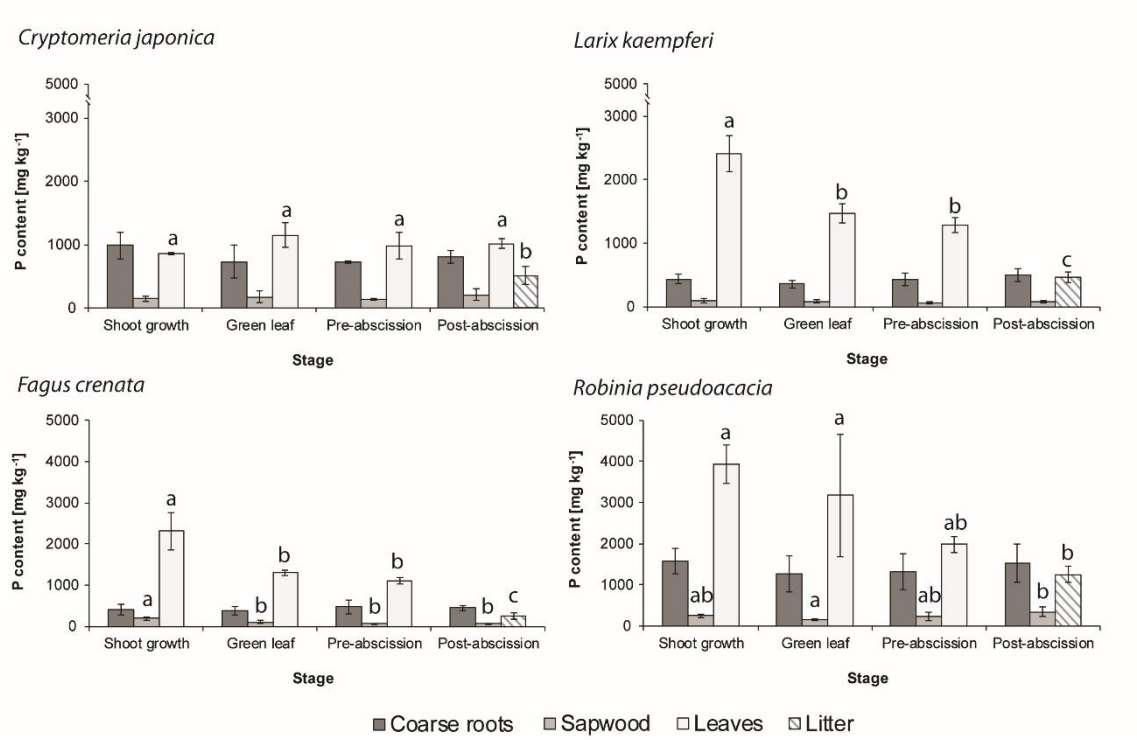


Figure 2: Seasonal pattern of P content in coarse roots, sapwood and leaves of *Cryptomeria japonica*, *Larix kaempferi*, *Fagus crenata* and *Robinia pseudoacacia*. The error bars denote SD (n=3 per species), letters indicate significant differences (min $P < 0.05$) among the same tissue.

Regardless of tree species, P content in leaves decreased significantly, before leaves were abscised (Fig. 2). Leaf P content was highest in *Robinia pseudoacacia* and *Larix kaempferi*, while *Fagus crenata* and *Cryptomeria japonica* had the lowest P content ($P < 0.05$) during the whole year. Furthermore, *Cryptomeria japonica* leaves showed no significant differences in P content among age groups (Fig. 3). In general, *Larix kaempferi* was most efficient in reabsorbing P, followed by the broad-leaved species, while *Cryptomeria japonica* was least efficient, which is reflected in their PRE (Table 4). However, both coniferous tree species were more proficient in reabsorbing P than the broad-leaved species.

When comparing all plant tissues, we can observe a common pattern across all species at any phenological stage, where the lowest P content was found in the sapwood followed by coarse roots and the highest P content was found in leaves.

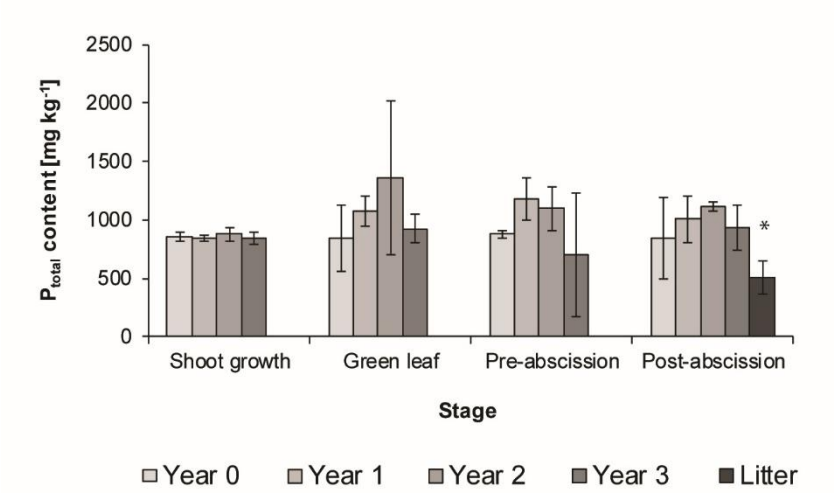


Figure 3: Seasonal pattern of P content in leaves of *Cryptomeria japonica* separated by leaf age (n=3 per leaf age class). Asterisks indicate significant differences ($P < 0.05$).

Table 4: N and P resorption efficiency (NRE and PRE, respectively), their corrected value to leaf weight loss during reabsorption (NRE* and PRE*, respectively) and N and P proficiency NRP and PRP, respectively) of *Cryptomeria japonica*, *Larix kaempferi*, *Fagus crenata* and *Robinia pseudoacacia* with ± denoting SD. N related values are based on n=9 trees per species, P values are based on n= 3 trees per species.

Species		NRE [%]	NRE* [%]	NRP [% dry mass]	PRE [%]	PRE* [%]	PRP [% dry mass]
Cryptomeria japonica	0-year old leaf	49 ± 9^{a}	63 ± 6^{a}	0.6 ± 0.1^{a}	55 ± 4^{ac}	66 ± 3^{ac}	0.05 ± 0.01^{a}
	3-year old leaf	34 ± 3^{1bd}	51 ± 7^{bd}				
Larix kaempferi		36 ± 13^{bd}	52 ± 10^{bd}	0.9 ± 0.1^{b}	68 ± 7^{abc}	76 ± 5^{abc}	0.05 ± 0.01^{a}
Fagus crenata		67 ± 4^{c}	74 ± 3^{c}	0.7 ± 0.1^{ac}	81 ± 5^{b}	85 ± 4^{b}	0.03 ± 0.01^{a}
Robinia pseudoacacia		32 ± 12^{d}	47 ± 10^{d}	2.6 ± 0.2^{d}	52 ± 16^{c}	63 ± 11^{c}	0.13 ± 0.01^{b}

Letters indicate significant differences ($P < 0.05$)

3.3 P cycling on whole-tree level

In general, coarse roots and sapwood of all tree species did not change significantly throughout the phenological stages with the exception of the sapwood of *Robinia pseudoacacia* increasing ($P < 0.05$) in P content from the pre- to the post-abscission stage (Table 5). Based on the non-significant changes of P content in coarse roots and sapwood, we calculated on whole-tree level that *Fagus crenata* and *Robinia pseudoacacia* reabsorbed more ($P < 0.05$) P than *Larix kaempferi* and *Cryptomeria japonica*. The amounts of reabsorbed leaf P stored in plant tissues formed 27% of P storage in *Larix kaempferi*, while 73% came from another source. For *Fagus crenata*, we found that P stored in coarse roots was similar to P released from sapwood while leaf P reabsorption was 16.8g P. *Robinia pseudoacacia* stored almost 40% of reabsorbed leaf P in coarse roots and sapwood leaving 60% unaccounted for. Evergreen *Cryptomeria japonica* resorbed the lowest amount of P from to-be-abscised leaves on whole-tree level. However, considering the remaining living leaves, we observed P reabsorption towards woody storage tissues as well. Sapwood and roots together seemed to store 25.8 g P at whole tree level, possibly made up of 4.3 g P reabsorbed from leaves falling as litter and 20 g P from remaining living leaves. Thus, total leaf P reabsorption from dead and living leaves added up to 24.3 g P making this evergreen species resorbing the highest P amounts from leaves compared to the deciduous species, forming 74% of whole-tree P storage and consequently 26% must have derived from another source.

Table 5: Biomass during the green leaf stage and seasonal change in whole-tree P content, total leaf P reabsorption and P storage per plant tissue of *Cryptomeria japonica*, *Larix kaempferi*, *Fagus crenata* and *Robinia pseudoacacia* (n=3 per species) with ± denoting SD. Significant differences among same plant tissues of the same species are highlighted in bold.

Species	Tissue	Biomass [kg]	P [g P]			P reabsorbed from leaves [g P]	Stored P [g P]
			Green leaf stage	Pre-abscission stage	Post-abscission stage		
Cryptomeria Japonica	roots	128.3 ± 17.1	94.0 ± 36.7	92.9 ± 10.7	104.9 ± 23.9	-	15.3 ± 21.4
	sapwood	321.0 ± 68.6	60.9 ± 45.33	44.0 ± 15.0	71.4 ± 44.5	-	10.5 ± 5.0
	leaves / litter	101.4 ± 20.7	113.7 ± 13.1	99.3 ± 15.4	93.7 ± 3.4 **/ 3.4 ± 0.6****	4.3 ± 0.8	-20 ± 12.6
Larix kaempferi	roots	129.0 ± 37.7	45.1 ± 16.6	57.1 ± 23.5	62.8 ± 14.6	-	17.7 ± 8.2
	sapwood	105.0 ± 23.9	8.5 ± 4.1	6.0 ± 2.9	7.4 ± 1.8	-	-1.0 ± 4.2
	leaves / litter	4.0 ± 0.8	6.6 ± 1.9	5.7 ± 1.6	- **/ 2.1 ± 0.6****	4.5 ± 1.3	-
Fagus crenata	roots	111.6 ± 3.9	38.2 ± 9.6	53.0 ± 20.3	50.4 ± 6.4	-	12.5 ± 10.6
	sapwood	349.1 ± 8.9	35.1 ± 11.2	21.5 ± 7.2	21.0 ± 2.6	-	-14.1 ± 13.7
	leaves / litter	8.7 ± 0.2	11.3 ± 0.5	9.6 ± 0.8	- **/ 2.2 ± 0.7****	9.2 ± 2.9	-
Robinia pseudoacacia	roots	6.0 ± 0.2	7.7 ± 3	8.0 ± 2.8	9.6 ± 2.9	-	1.9 ± 2.9
	sapwood	8.0 ± 0.4	1.2 ± 0.2	1.9 ± 1.0	**2.7 ± 1.0***	-	1.5 ± 1.2
	leaves / litter	4.8 ± 0.9	18 ± 8.8	9.6 ± 2.5	- **/ 5.8 ± 0.3***	8.8 ± 0.4	-

***P* < 0.05** ****P < 0.01**

3.4 N and P cycles interaction in leaves

All species reabsorbed N and P from leaves (Table 6), with the highest NRE observed for *Fagus crenata* ($P < 0.05$), followed by *Cryptomeria japonica* and *Larix kaempferi,* while *Robinia pseudoacacia* showed the lowest efficiency. Similar to NRE, *Fagus crenata* showed the highest PRE ($P < 0.05$), followed by *Larix kaempferi, Cryptomeria japonica* and *Robinia pseudoacacia* being least efficient (Table 4). Further, the non-leguminous tree species were the most N and P proficient with similar values, while *Robinia pseudoacacia* was the least proficient ($P < 0.01$). If the N : P ratio is lower than 14, plants are assumed to grow under N limited conditions, while values over 16 mean that they grow under P limited conditions. If the values are between 14 and 16, plants are co-limited in both N and P (Koerselman & Meuleman, 1996). During the shoot growth stage, all species were under N limited conditions with values lower than 14(Table 6). Throughout all phenological stages, leaves of *Cryptomeria japonica* remained P limited even after abscission. *Larix kaempferi* became gradually less limited by N but was never N and P co-limited. Only after leaf abscission, litter was P limited reflecting this species difference in NRE and PRE. Leaves of *Fagus crenata* shifted from the shoot growth to the green leaf stage towards N and P co-limitation but during the pre-abscission period leaves were N limited again. After leaf abscission, litter *of Fagus crenata* was most P limited following their NRE and PRE. Finally, leaves of *Robinia pseudoacacia* showed clear shift of the N : P ratio from N limitation to first N and P co-limitation during the green leaf phase and increasing towards P limitation along the remaining phenological stages. Only *Cryptomeria japonica* reabsorbed N and P in similar proportions.

Simple linear regression analysis of leaf N and P content of all phenological stages revealed more details about the relationship between N and P in the four tree species: *Larix kaempferi*, and *Robinia pseudoacacia* had a similar proportion of N to P increase, while *Fagus crenata* and *Cryptomeria japonica* increased more in N than P (Fig. 4).

Further, looking at each individual species the R^2 was highest in *Fagus crenata* followed by *Larix kaempferi* and *Cryptomeria japonica*, and *Robinia pseudoacacia*.

Table 6: Mean ± SD of leaf phosphorus and nitrogen content and N : P ratio of *Cryptomeria japonica*, *Larix kaempferi*, *Fagus crenata* and *Robinia pseudoacacia* (n=3 per species). Significant differences among same tree species are highlighted in bold.

Species	Phenological stage	P [mg kg^{-1}]	N [mg kg^{-1}]	N : P ratio
Cryptomeria Japonica	shoot growth	857 ± 15	8348 ± 238	9.7 ± 0.1
	green leaf	**1150 ± 191***	**11760 ± 225***	10.5 ± 2.0
	pre-abscission	986 ± 208	**11151 ± 1184***	11.4 ± 1.7
			living leaf / litter	
	post-abscission	1019 ± 75 / **512 ± 140****	**11372 ± 1726*** / **5606 ± 541***	11.2 ± 0.6 / 10.0 ± 1.5
Larix kaempferi	shoot growth	2410 ± 284	18174 ± 2999	7.6 ± 1.3
	green leaf	**1471 ± 152****	**14440 ± 1610****	9.8 ± 0.8
	pre-abscission	**1286 ± 114****	**16122 ± 921****	**12.6 ± 1.8***
	post-abscission	**468 ± 87****	**9370 ± 1030****	**20.2 ± 1.8****
Fagus crenata	shoot growth	**2310 ± 451****	23713 ± 3535	10.3 ± 0.7
	green leaf	1307 ± 67	21614 ± 2554	16.6 ± 2.4
	pre-abscission	1105 ± 87	**13748 ± 3243****	12.3 ± 1.9
	post-abscission	**253 ± 82****	**6634 ± 713****	**27.7 ± 7.5****
Robinia pseudoacacia	shoot growth	3930 ± 468	38371 ± 4139	9.9 ± 2.1
	green leaf	3174 ± 1494	42672 ± 4152	14.9 ± 4.5
	pre-abscission	**1986 ± 194***	31834 ± 2680	16.2 ± 2.8
	post-abscission	**1250 ± 198****	**26820 ± 2704***	**21.6 ± 2.2****

***P* < 0.05** ****P < 0.01**

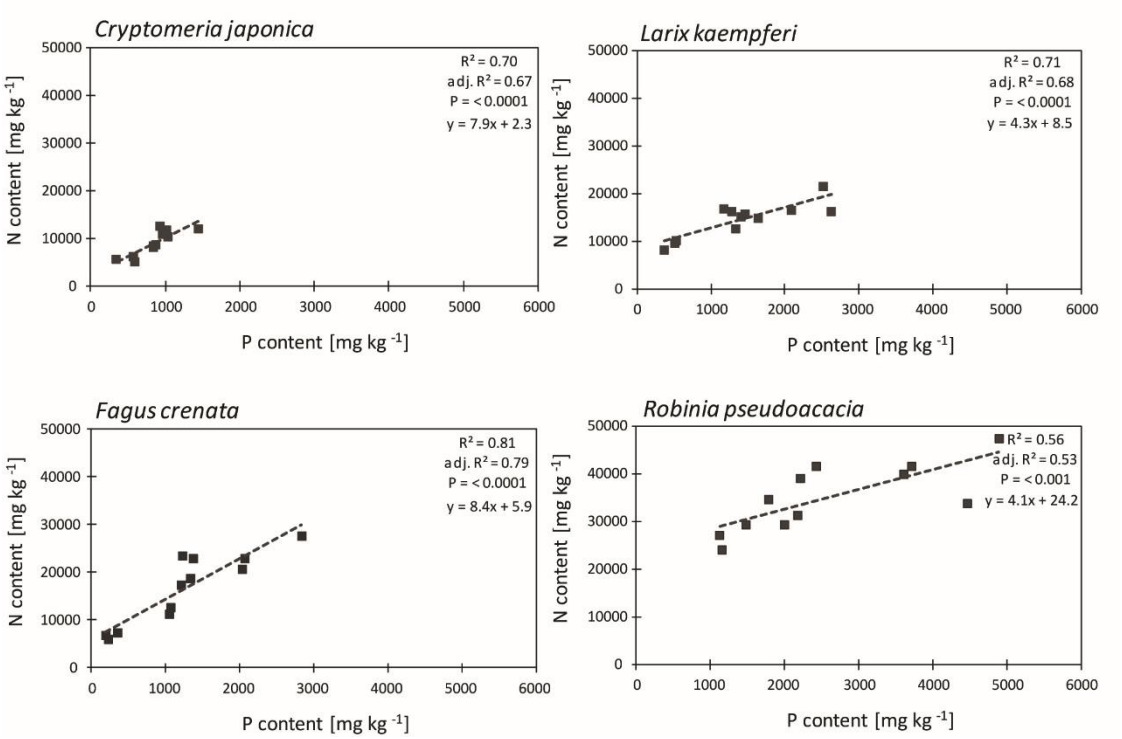


Figure 4: Results of simple linear regression analysis of nitrogen and phosphorus content in *Cryptomeria japonica*, *Larix kaempferi*, *Fagus crenata* and *Robinia pseudoacacia* (n=3 per species).

4 Discussion

4.1 N & P cycling in the soil

One main difference between N and P in forest ecosystems is their source: various forms of N (e.g. ammonium, nitrate, dissolved organic N) can be soil-derived or uptaken from the atmosphere by N-fixing bacteria (Tian et al., 2003), while P originates exclusively from the base rock and is taken up in the form of inorganic phosphate (Plassard & Dell, 2010; Chiou & Lin, 2011; Rennenberg & Herschbach, 2013). In general, the N and P cycles are linked in the soil, where microorganisms and plants produce enzymes that mineralize organically bound nutrients (Olander & Vitousek, 2000; Turner, 2008). P demand and P mineralization form a strongly regulated cycle by production of these enzymes when P demand exceeds P mineralization resulting in a higher P mineralization. If sufficient organic P is available in the soil, plant P demand will be mostly met by P mineralization but never exceed it. In contrast, N mineralization may exceed plant demand ultimately leading to N saturation, as the

regulatory cycle between plant N demand and N mineralization is weaker than the one for P, as incidental mineralization of N occurs during decomposition processes (C mineralization) (Olander & Vitousek, 2000). The soil total and available P content of our study combined with the total and available N characteristics of the same samples taken from Seidel et al. (2019a, b, c) showed that *Larix kaempferi* grew on the N poorest soils with the lowest available P among all species. This indicated poorer N & P mineralization rates in the *Larix kaempferi* stands in comparison to the other tree species (Pastor et al, 1984).

4.2 N & P cycling of Cryptomeria japonica

Figure 2 showed that the evergreen *Cryptomeria japonica* had on average throughout the seasons the highest P content in coarse roots and sapwood and low P content in leaves in comparison to the same plant tissues of the other non-leguminous species in this study. This indicated that this evergreen species seemed to have a greater need for P in its woody tissues in comparison to deciduous trees, which has also been found for evergreen *Quercus oleoides* (Waring et al., 2015). At the same time, N content of coarse roots and sapwood was similar to *Larix kaempferi* and *Fagus crenata* while having the lowest leaf N content among all species (Fig. 5). Considering that this species had a low PRE and a modest NRE as well as a low PRP and the lowest NRP with a N : P ratio of around 10 along the season, as this tree maintains a stable N and P content and was the least N limited non-leguminous species in this study. The whole-tree analysis revealed that roots and sapwood tended to store P and that this storage might be mostly made up of leaf reabsorbed P indicating that only minor amounts of soil P were stored in these tissues. Additionally, P may be stored in buds, twigs, branches and bark (Chapin & Kedrowski, 1983; Rosecarnce, 1998; Son et al., 2000), which would suggest a higher soil P uptake and storage during the pre-abscission stage which should be addressed in future studies. On whole-tree level, this species has allocated the highest amounts of P as well as N (Seidel et al., 2019a) in its tissues during all phenological stages even if compared to *Fagus crenata* trees of similar age (Seidel et al., 2019b). Further, our NRE and PRP values are in agreement with Son et al. (2000) for the evergreen *Pinus rigida* and for NRE with Enta et al. (2019) and Kobayashi & Tashiro (2003) for *Cryptomeria japonica*. Being an evergreen, P was not only reabsorbed from shedding leaves, but also from the large amount of remaining living leaves, increasing the total amounts of P

reabsorbed from leaf tissues making it essentially the tree species resorbing the most P from leaves to storage tissues among the studied tree species. However, this is in contrast to Chapin & Kedrowski (1983) who found that evergreen spruce (*Picea mariana*) stored P mainly in leaves of different taiga trees. Linear regression showed that *Cryptomeria japonica* increased less in P content per increase in N content than *Larix kaempferi* and *Robinia pseudoacacia* suggesting a possibly higher photosynthetic P-use efficiency as it is typical for fast growing tree species like *Cryptomeria japonica* (Son et al., 2000; Gan et al., 2015). Thus, with expected anthropogenically increased N deposition as it has been observed in urban areas around the world (Templer et al., 2007; Fukushima et al., 2011; Schmitz et al., 2019), this tree might tolerate P limitation longer than the other tree species. *Cryptomeria japonica* appeared to allocate nutrients and follow a more nutrient conservative strategy, which makes it less sensitive to nutrient availability changes than the other species. In forests prone to N saturation, it would be advisable to promote the planting of *Cryptomeria japonica* as it seemed to have a high P use efficiency and was very N and P proficient. Further, application of N fertilizer in autumn might be beneficial to increase N storage in roots and sapwood.

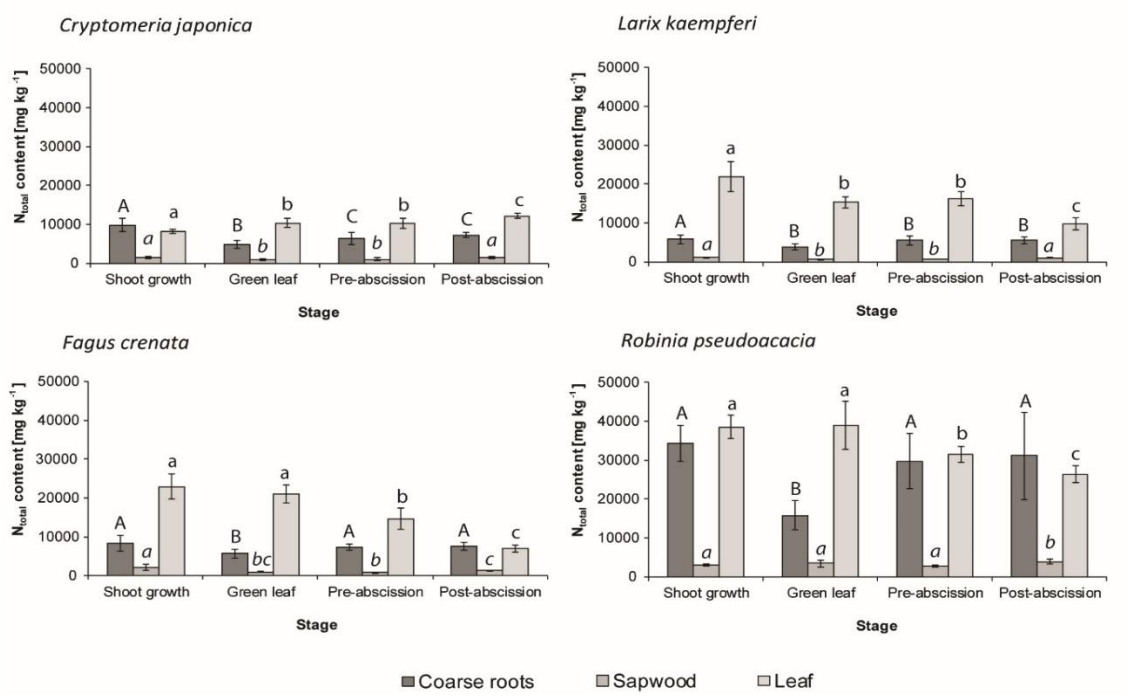


Figure 5: Seasonal pattern of N content in coarse roots, sapwood and leaves of *Cryptomeria japonica*, *Larix kaempferi*, *Fagus crenata* and *Robinia pseudoacacia*. The error bars denote SD (n= 9 per species), letters indicate significant differences (min $P < 0.05$) among the same tissue. Taken from Seidel et al. (2019a, b, c).

4.3 N & P cycling of Larix kaempferi

This species showed low N and P contents in all plant tissues throughout all phenological stages among the species of this study while growing in the nutrient poorest soil. Further, this tree exhibited a high PRE and a low PRP while having a low NRE and a middling NRP, suggesting that this tree rather conserves P over N. This is supported by the shift of the N : P ratio from N limited conditions during the shoot growth stage to P limitation during leaf N and P reabsorption. The values found for leaf P content in our study were in agreement with Li et al. (2016) and Son et al. (2000) reported similar NRE and PRE values for *Larix leptolepsis*. The NRE of *Larix kaempferi* reported by Enta et al (2019) was higher (50.9 %) than ours but they did not investigate the soil conditions, which may be a reason for this difference (Olander & Vitousek, 2000). The whole-tree measurements demonstrated that roots tended to act as P storage with no contribution of the sapwood. As leaf P reabsorption was not sufficient to fill this storage, substantial amounts of P uptaken during the pre-abscission stage may have contributed significantly to P storage. This value might be even higher, as buds, twigs and bark may be possible P storage tissues as well (Chapin & Kedrowski, 1983; Rosecarnce, 1998; Son et al., 2000). On whole-tree level this species P content is middling but its N content is lowest among these species (Seidel et al, 2019b). These insights presented *Larix kaempferi* as a rather low nutrient demanding tree that is able to grow well in poor soil nutrient conditions. In forests, characterized by very poor N and P availability, *Larix kaempferi* can thrive and as it is a fast growing species like *Cryptomeria japonica*, planting of this tree could increase C fixation better than the other species of this study. Application of N or P fertilizer to this species seems unnecessary as it grows well in low nutrient environments. However, if fertilization is conducted, we recommend adding rather P than N.

4.4 N & P cycling of Fagus crenata

Fagus crenata plant tissues showed low P and N content in coarse roots and sapwood while leaf N was high in comparison to the non-leguminous species and P was rather low in comparison to *Larix kaempferi* and *Robinia pseudoacacia* with the highest PRE and NRE. Enta et al. (2019) and Yasumura et al. (2005) reported a similar NRE value for *Fagus crenata*. Additionally, PRP and NRP were low and the leaf N : P ratio along the gradient of N limitation

and N and P co-limitation throughout the phenological stages where finally the abscised leaves were P limited. This suggested that a) P was better plant internally recycled than N and b) N was most limiting during the shoot growth stage among all species c) a decrease in P supply may cause N and P co-limitation throughout all phenological stages.. Further, whole-tree measurements revealed that P was reabsorbed from leaves and sapwood and stored in coarse roots. The net balance of these values exposed that there must be another major N storing plant tissue which might be twigs and/or branches and/or bark as shown for *Pistacia vera* (Rosecarnce 1998), *Pinus rigida* and *Larix leptolepis* (Son et al., 2000). Additionally, the biomass data of this tree species indicated on whole-tree level a concentration of nutrients rather in the roots than aboveground plant tissues especially for N (Seidel et al., 2019b). The results suggested that this tree species is N and P demanding but the same time highly efficient reabsorbing N and especially P, with the latter being reported for deciduous trees in general in comparison to evergreen coniferous species (Ishida et al., 2006; Kang et al., 2010). Higher N deposition in *Fagus crenata* forests might initially lead to enhanced growth, photosynthetic activity and P mineralization. However, these forests may become P limited, as this species was already close to N and P co-limitation during the growing season.

4.5 N & P cycling of Robinia pseudoacacia

Robinia pseudoacacia was the only leguminous species in this study, and thus it was independent from soil N uptake and richer in N and P in all plant tissues. Due to this abundance of N, this species showed the lowest NRE and NRP as well as the highest NRP and PRP in comparison to the other species. All the same, this species was still N limited during the shoot growth stage as the other species were. Yet, throughout the phenological stages, this N limitation shifted gradually towards N and P co-limitation during the green leaf and pre-abscission stage. The N : P ratio of the litter demonstrated the dependence of this species on recycling P more efficiently. Whole-tree measurements revealed that P was stored in roots and sapwood but leaf reabsorbed P exceeded the storage capability of these tissues, thus other plant tissues must act as an additional plant storage, such as twigs and/or branches and/or bark (Chapin & Kedrowski, 1983; Rosecarnce, 1998; Son et al., 2000). The whole-tree comparison of these still very young trees with the other species is not ideal, as

these were adult trees, thus measurements of adult *Robinia pseudoacacia* trees in the future are necessary to confirm our findings as especially the asymmetrical growth of coarse roots and sapwood may shift their respective importance for P storage (Blujdea et al., 2011). Further, Enta et al. (2019) reported a lower NRE (18%) for the same research plot and a similar NRP while the values found in our study are similar to those found by Lima et al. (2006), Singh (2014 and 2015). This difference might be linked to differences in climatic conditions of the sampling years as well as differences in sampling time. However, our study showed clearly the strong effect of soil N independency on P content in this leguminous tree species following an exploitative nutrient strategy, enabling it to acquire the highest amounts of N and P, supporting rapid growth and being a soil N and P fertilizer due to its low PRP and NRP (Lopez et al, 2014). In order to promote its growth, N fertilizer applied in spring and P fertilizer applied in autumn could be beneficial, as throughout the season, this species shifts from N limitation to P limitation. As this tree usually is an invasive species, we do not recommend promoting its growth with fertilizer as it reduces biodiversity strongly (Vítková et al., 2017). If atmospheric N increases, *Robinia pseudoacacias* growth and photosynthetic activity may increase during the shoot growth and green leaf stage but the moment of P limitation along the seasons might shift to an earlier phenological stage.

4.6 General remarks about tree species P cycling

As plants require P-rich ribosomal RNA to initiate growth, we could observe the highest leaf P concentrations in the early growing season (Li et al., 2017) with *Cryptomeria japonica* as the exception. This may be linked to the timing of sampling, while the other tree species needed to start their leaf growth from a bare crown, evergreen *Cryptomeria japonica* had already leaves and thus this initial growing phase with a peak in leaf P content may have already concluded. Furthermore, compared with deciduous trees, evergreen trees tended to have lower leaf N & P concentrations, as their photosynthetic rates per mass are generally lower (Ishida et al., 2006). The next step to further clarify the P cycle is to gain insights of other tree species to identify distinct traits and patterns besides the four species that were presented here. Additionally, analysis of bark, branches and twigs is needed to clarify their role as possible P storage tissues and consequently identify the different processes involved in P storage.

5 Conclusion

To our knowledge, the present study was the first to try to quantify P storage pools in leaf, root and sapwood and related them to N dynamics along four phenological stages in typical Japanese forest ecosystems. Based on the results obtained in this study, we concluded that evergreen *Cryptomeria japonica* seemed to follow a nutrient conservative strategy that may enable this tree species to be more tolerant towards nutrient availability changes than the other studied species. Deciduous *Larix kaempferi* was a low N and P demanding tree that was able to grow well in poor nutrient conditions while broad-leaved *Fagus crenata* was highly N and P demanding, while highly efficient reabsorbing N and P. Finally, leguminous *Robinia pseudoacacia* followed and a nutrient exploitative strategy demonstrating clearly the effect of soil N independence on plant tissues P content, enabling it to acquire the highest amounts of N and P. The results of this study showed the particular interaction of N and P inherent to each tree species and shed new lights on different tree species nutrient cycles that we can use to improve forest management practices.

Acknowledgements

We want to thank the staff of the University Research Forest of Yamagata University for keeping the site accessible in summer and winter. We also thank Cristina Lerda from the Università degli Studi di Torino, for the assistance measuring available P in soil.

Author contributions

Felix Seidel and M. Larry Lopez C. have done the conceptualization and methodology for this study. Felix Seidel conducted the investigation, formal analysis, data curation & visualization and prepared the original draft of the manuscript. M. Larry Lopez C. was supervising this study. M. Larry Lopez C., Eleonora Bonifacio, Luisella Celi and Hiroko Kurokawa contributed to data interpretation, writing, and reviewing of the manuscript.

Chapter 6

Soil fungal community differences between four Japanese cool-temperate forest tree species

Felix Seidel[1], Carles Castaño[2], Josu G. Alday[3], M. Larry Lopez C.[1], José Antonio Bonet[3]

[1]Faculty of Agriculture, Yamagata University, Tsuruoka, Japan

[2]Department of Forest Mycology and Plant Pathology, Uppsala BioCenter, Swedish University of Agricultural Sciences, SE-750 07, Uppsala, Sweden

[3]Departament de Producció Vegetal i Ciència Forestal, Universitat de Lleida-AGROTECNIO, Av. Rovira Roure, 191, 25198 Lleida, Spain

Author for correspondence:

Felix Seidel

Tel.: +81-235-28-2961

E-Mail: seidel.geo-science@gmx.de

Faculty of Agriculture Yamagata University

Tsuruoka-shi, Wakabamachi 1-23, 997-8555

Yamagata Prefecture, Japan

Abstract

Background and Objectives: Over 70% of Japan is covered by forest and the understanding of nutrient cycling and carbon sequestration is crucial in order to manage these forests efficiently. Soil nutrients in forests and associated fungal communities are key drivers of vegetation structure. However, information on soil fungal diversity and composition in Japanese forests is scarce. The aim of this study was to describe the soil fungal communities and their relationship with soil nutrient status across different forest types.

Materials and Methods: Four different forest types: *Cryptomeria japonica*, *Larix kaempferi*, *Fagus crenata* and *Robinia pseudoacacia* were selected to study the soil fungal communities. 31 soil samples were taken at two different depths, and fungal communities were identified by DNA sequencing of the ITS2 region. We studied the correlation between soil fungal community structure and composition with soil variables, and how this relates to vegetation composition.

Results: Soil fungal communities were tree species dependent, varying slightly with soil depth. Mycorrhizal fungi were abundant under *Larix kaempferi* and *Fagus crenata* plots, while almost no presence of these taxa was detected in *Cryptomeria japonica* and *Robinia pseudoacacia* plots. However, taxonomy of many species remained unknown, especially in *Cryptomeria japonica* plots (51%). pH, nitrate content and C/N ratio significantly were related to (34%) the fungal community structure. Our data suggested that C-sequestration was possibly higher in *Cryptomeria japonica* and *Robinia pseudoacacia* plots than in the other tree species.

Conclusions: We suggest favouring the planting *Cryptomeria japonica* over *Fagus crenata* and *Larix kaempferi* in order to reduce atmospheric C by binding C more efficiently in forest soils. *Robinia pseudoacacia* showed similar C sequestration capabilities as *Cryptomeria japonica*, but as it is an invasive species and reduces biodiversity considerably, we do not recommend planting it. The next steps consist in taxonomically identify the large number of unknown fungal species found in this study, especially for *Cryptomeria japonica,* in order to improve our understanding of the nutrient cycling of these typical tree species. This in turn will allow us to adjust best management practices.

Keywords: *Cryptomeria japonica*, Ectomycorrhiza, *Fagus crenata*, *Larix kaempferi*, *Robinia pseudoacacia,* DNA, Fungi.

1 Introduction

The understanding of nutrient dynamics and carbon sequestration are crucial in order to manage forests ecosystems efficiently and maintaining the ecosystem services that they provide. Nutrient dynamics of forest are highly affected by soil bacteria, fungi and other soil microorganisms (De Bellis et al., 2007). Among soil biota, soil fungi, which account for the majority of the soil microbial biomass, are essential drivers of many ecosystem processes (Lin et al., 2016; Castaño et al., 2018a). Soil fungi decompose tree litter, break its components down into plant available nutrients (Dighton, 2003) and indirectly regulate carbon sequestration and nutrient dynamics (Fontaine et al., 2010). Among fungi, mycorrhizal species are one of the most important soil microbiome functional groups helping their host plants to uptake water and nutrients (Smith & Read, 2008; Cairney, 2012). Especially, nitrogen (N) has strong effects on ectomycorrhizal fungi (EMF) formation, as EMF are considered as an adaptation to nutrient limited conditions (Hobbie & Agerer, 2010) and changes in soil N availability can alter the EMF community. Under increased N deposition, reliance of host trees on EMF for nutrient supply decreased for fungi that form a symbiosis with a wide range of tree species. However, these generalist species seemed to be less affected by increased N deposition than more specialised EMF species (Wallenda & Kottke, 1998). Many industrialised countries experience an increase in atmospheric N deposition possibly leading to N saturated forest ecosystems, as it has been already observed in Europe and America (Templer et al., 2007; Peri et al., 2012; Schmitz et al., 2019), as well as in Asia (Fang et al. 2009; Takebayashi et al., 2010; Fukushima et al., 2011) but the implications of this are far from complete.

Another important fungal functional group are saprotrophs, which are crucial for litter decomposition (Baldrian et al., 2011). The soil fungal community composition and abundance of both EMF and saprotrophs have important consequences for nutrient cycling, determining plant community structure (Sardans et al., 2013). However, while soil fungal communities may be the major drivers of plant community structures and forest biodiversity, plant species diversity is also strongly influencing fungal community structures (Windler & Shamoun, 2006; De Bellis et al., 2007), representing a feed-back mechanism (Clemmensen et al., 2015). De Bellis et al. (2007) found that most variation in micro fungal communities was explained rather by plant species composition than by differences in soil

chemistry demonstrating the importance of litter supply and quality subsequently influencing e.g. the structure of decomposer communities (Conn & Dighton, 2000). However, fungal community structure is also affected by other biotic and abiotic factors such as climate (O'Dell et al.,1999), soil type (Moser et al., 2005; Walker et al., 2005), soil nutrient status (Nantel & Neumann, 1992), EMF succession (Nara et al., 2003) and interspecific integration of EMF (Koide et al., 2005). Further, decreases of soil fungal species richness and fungal biomass with soil depth have been reported previously in boreal and temperate forest ecosystems (Jumpponen et al., 2010; Andreetta et al., 2011; Voříšková et al., 2014), which are related to changes in nutrient quality and abundance. However, most of the studies are only focused on the humus and top soil layer, thus information about changes with deeper mineral horizons is still lacking.

In general, information on soil fungal communities in Japanese forests is scarce and limited to the Kanto region (Nara, 2006; Taniguchi et al., 2007; Ochimaru & Fukuda, 2007; An et al., 2008; Miyamoto et al., 2014) and studies conducted in cool temperate forests of Japan are exiguous (Ishida et al., 2007; Fukasawa et al, 2009).

Therefore, a thorough identification of fungal communities and their changes in composition with soil depth among different host species within the same environmental conditions is the first step to elucidate effects of hosts on fungal communities of Japanese cold temperate forests (Ishida et al., 2007). The aim of this study was to describe the fungal community composition in two soil depths and their relationships with soil nutrient status across four different host species. We hypothesized that changes in fungal composition with host species and fungal species richness and abundance decreases with increasing soil depth, following the pattern found between humus and top soil layer (Jumpponen et al., 2010; Andreetta et al., 2011; Voříšková et al., 2014). The aims were (1) to identify soil fungal compositional differences between host species, (2) to correlate fungal community structure with soil nutrient status and (3) thus, to get indirectly insights into the carbon sequestration capabilities of these different forest types.

2 Materials and Methods

2.1 Study site

The sampling sites are located in north-eastern Japan in the Research Forest of Yamagata University (N38° 32.889' E139° 51.706'). The sites elevation ranged from 300 to 700 m above sea level. The climate is humid with an annual precipitation of 3000 mm, and approximately half of the precipitation falling in form of snow, covering permanently the sampling sites from December to May.

The sampling sites were the same as in Seidel et al. (2019a, b, c) and chosen by accessibility, stand age and purity within a maximum distance of 3 km to guaranteeing comparability of the measured soil parameters and the soil sampling of this study. In total 10 plots were established over four forest types.

Three plots of Japanese cedar (*Cryptomeria japonica* D. Don.) were established in 50-year old pure plantations with an understory vegetation consisting of broad-leaf bamboo (*Sasa veitchii*) at the lowest elevation of 300 m above sea level. For Japanese larch (*Larix kaempferi* Lamb.), since there were no pure stands in the Yamagata University research forest, we chose three plots from a mixed forest (two species cohabiting), but dominated by 40-50-year old *Larix* trees (~80-90%). Three pure stands of 70-80 year old Japanese beech (*Fagus crenata* Blume) growing at the highest elevation (~700 m above sea level) were selected as well as a single plot of 7-year old Black locust (*Robinia pseudoacacia*), as it is the only site in the Yamagata University forest where it grows in a pure stand. This area was once covered by a Japanese cedar plantation, which was harvested and subsequently burned. After the fire, *Robinia pseudoacacia* colonized the plot.

2.2 Sample collection and treatments

Soil pits in each plot near the sampled trees were dug and soil horizons identified in the field following the FAO guidelines (FAO, 2006) and genetic horizons were sampled for soil characterization as described in Seidel et al. (2019a, 2019b, 2019c).

For soil fungal community analysis, we sampled soil around 31 tree individuals on May 2018. Four tree species were sampled: i) three individuals per site for *Cryptomeria japonica* and *Fagus crenata* were sampled (n= 9 per species) ; ii) eight individuals were sampled for *Larix*

kaempferi (total n= 8), as we were not able to find nine accessible trees of same age, and iii) 5 individuals of *Robinia pseudoacacia* were sampled in one site (n= 5).Around each tree, 4 opposite points were sampled at two different depths: 0-5 cm (Ah horizon) and 5-15cm (Bv horizon). All soil samples were taken from the field in a cooler box and freeze dried for at least 48h and then sieved (<2 mm). Subsequently, the samples of each individual tree were pooled (4 samples, each 3.5g), homogenized and stored in a freezer until DNA extraction.

2.3 DNA extraction & amplification

Genomic fungal DNA was extracted with the NucleoSpin® soil kit (Macherey-Nagel, Duren, Germany) following the manufacturers protocol. DNA was extracted from 0.350 mg of soil with 900µl lysis buffer (SL1) due to the dryness of the samples.

The ITS2 region was amplified with a Mastercycler® nexus X2 (Eppendorf, Germany). gITS7 and ITS4 primers (Castaño et al., 2018) were used. They have been fitted with unique 8-bp tags, differing in at least three positions. Each individual sample was tested in the PCR to determine the optimum number of cycles for amplification. Most samples amplified well between 26 and 28 cycles. Samples were amplified in triplicates with negative controls of DNA extraction and PCR. Final concentrations in the 50 µl PCR samples were: 25ng template, 200 µM of each nucleotide, 2,75 mM $MgCl_2$, Primers, at 200 nM, 0.025 U μL^{-1} polymerase (DreamTaq, Thermo Scientific, USA) in 1X buffer PCR. PCR conditions were as follows: 5 min at 95°C, followed by 26-28 cycles 30 s at 95°C, 30 s at 57°C, 30 s at 72°C. Finally, 7 min at 72°C before storage at 4°C. These samples were purified with the AMPure kit (Beckman Coulter Inc., USA) and quantified using a Qubit fluorometer (Life technologies, USA). From each sample, equal amounts of DNA were pooled and purified using the ENZA Cycle Pure kit (Omega Biotek). Quality control of the purified samples was conducted using a BioAnalyzer 2100 (Agilent Technologies, USA) and a 7500 DNA chip. The DNA library was sequenced using the Illumina MiSeq platform, with 300-bp paired-end read lengths, generating 1.5 M sequences.

2.4 Bioinformatic analyses

The library was screened for quality control and sequence clustering using the SCATA pipeline (https://scata.mykopat.slu.se/). Internal Transcribed Spacer (ITS) sequences with <200 bp were removed, and remaining sequences were screened for primers (requiring 90% primer match) and sample tags. Sequences were also quality filtered, removing data with an average quality score < 20 or with a score < 10 at any position, using the amplicon quality option. Sequences were pair-wise compared using 'usearch' (Edgar, 2011) after collapsing homopolymers to 3 bp. Pairwise alignments were scored using a mismatch penalty of 1, gap open penalty 0 and a gap extension penalty 1. We used the species hypothesis (SH) concept to cluster the sequences (Kõljalg et al., 2013) using single linkage clustering method, with a requiring maximum distance of 1.5% to the closest neighbour to enter clusters. SHs found in negative controls were excluded from further analysis, together with singletons and sequences with tag jumps.

2.5 Taxonomic and functional identification

We identified the 600 most abundant species hypothesis found at the soil fungal community, which represented more than 90% of the total sequences. The most abundant sequences from each OTU was selected for taxonomic identification, using the massBLASTer implemented in PlutoF against the UNITE (Abarenkov et al., 2010). Taxonomic identities at species level were assigned using > 98.5% similarity. Functional identification was done by using FUNGuild (Nguyen et al. 2016).

2.6 Soil analysis for multivariate analysis

Soil texture was measured with a laser diffraction particle size analyzer (Coulter LS200 with an attached Fluid Module, Beckman Coulter GmbH, Germany), after treating the samples with H_2O_2 and $Na_4P_2O_7$. The analyses were triplicated. The pH was determined potentiometrically in a 1:2.5 soil : water suspension. The carbon (C) and N contents were analysed by dry combustion using SUMIGRAPH NC-220F automatic highly sensitive NC analyzer SCAS (Japan). The results were expressed on a dry-weight (105°C) basis.

For the determination of total N content and $\delta^{15}N$ in plant tissues, a Thermo Quest EA1110 Elemental Analyzer (Italy) connected to an IsoPrime (GV Instruments, UK) was used. The isotopic composition of samples was expressed relative to atmospheric N_2 ($\delta^{15}N$ = 0‰) on scales normalized to the known $\delta^{15}N$ values of laboratory working standards for glycine ($\delta^{15}N$ = −0.3), which was normalized to L-glutamic acid distributed as USGS-40 ($\delta^{15}N$ = −0.2‰) by SI Science Inc., Japan. Additionally a tertiary reference material was used, the in-house laboratory standard acetanilide (−0.89‰). The three working standards were analysed after every eight to ten samples during CF-IRMS runs to assess the replicability of the isotope measurements and normalization. One pulse of pure N_2 reference gas from a tank reservoir ($\delta^{15}N$ = −2.5‰) was discharged into the IRMS at the beginning of each chromatogram for both standards and samples. The accuracy obtained for standards and samples during the overall analytical procedure was better than ±0.2‰.

The results of $\delta^{15}N$ were expressed as ‰ deviation, relative to atmospheric N_2 ($\delta^{15}N$= 0 ‰):

$$\delta^{15}\mathrm{N} = \left(\frac{(^{15}N/^{14}N)_{sample}}{(^{15}N/^{14}N)_{standard}} - 1 \right)$$

Total dissolved N (TN_b) was extracted from the soil samples by shaking them with 50 ml of 1M KCl. The supernatant was centrifuged, filtered (0.45 µm) and stored in a refrigerator and analysed with a TOC/TN_b analyser (vario TOC cube, elementar, Germany) for TNb determination. Ammonium content was determined using the method of Crooke et al. (1971), whereas nitrate content was determined by using the method of Mulvaney (1996) modified by Miranda et al. (2001). The colorimetric determination of ammonium and nitrate content was conducted with a U-2000 Spectrophotometer (Hitachi, Japan). Dissolved organic nitrogen (DON) expressed in g kg^{-1} was calculated as follows:

$$DON = TN_b - (NH_4^+ + NO_3^-)$$

For plant materials, total phosphorous (TP) was extracted by the potassium peroxydisulfate degradation method. All samples were weighed out and burned at 550°C for 2 hours. Potassium peroxydisulfate was added followed by disintegration of all samples and standards in an autoclave (LSX-300 High pressure steam sterilizer, TOMY, Japan) for 1 hour at 121°C. The colour regent was added and after centrifugation (3000rpmm, 5min),

colourimetric determination of TP content was conducted with a microplate reader (Multiscan GO, Thermo Scientific, USA) measuring 300 µml of each sample and standard.

For soil samples, TP content was measured using the Bray extraction for exchangeable phosphate with detection by molybdate colorimetry. Samples were weighed out, the bray extraction solution was added and samples were shaking for 5 minutes followed by centrifugation (3000 rpm, 5 min) and filtration (Whatman filter 42, GE Healthcare Life Sciences, UK). Subsequently, Phenolphthalein, NaOH and the working reagent were added and after 12 min. the colourimetric determination of TP content of 300 µml of each sample and standard was conducted with a microplate reader (Multiscan GO, Thermo Scientific, USA).

Available phosphorus in soil samples was determined with the colourimetric P Olsen method (Olsen et al., 1954). 20 ml of Olsen extract was added to 1 g of dry soil (< 2 mm) and shaken with a horizontal shaking stirrer for 30 min followed by 5min of centrifugation at 3000 rpm. The supernatant was separated and 0.8ml of this solution were added to 0.2 ml of $H_2SO_4$2N and 0.4 ml of R1 and R2, respectively. The samples were then measured with a U-2000 Spectrophotometer (Hitachi, Japan).

All soil data has been previously published in Seidel et al. (2019a, b, c).

2.7 Statistical analyses

Statistical analyses were performed in the R software environment (v.3.4.2; R Development Core Team 2019), using the vegan package for multivariate and diversity analyses (Oksanen et al., 2018), and nlme package for linear and generalized mixed models (Pinheiro et al., 2013).

Soil fungi data was analysed using both multivariate and univariate methods. First, previous to any analysis, data was Hellinger-transformed. Second, the significance of soil community compositional differences between hosts (*Cryptomeria japonica, Larix kaempferi*, *Fagus crenata, Robinia pseudoacacia*) was tested using Permutational Multivariate Analysis of Variance (PMAV, 'adonis' function using Bray–Curtis distance). Here, an overall analysis was conducted with depth included as random factor, and after that, soil data was split in the

two soil layers (top vs. bottom). Similar results were obtained for both depths, thus, we decided do not split the depths in the rest of analyses. Third, nonmetric multidimensional scaling (NMDS, 'metaMDS' function with Bray–Curtis distance) was used to identify the understory compositional differences between the four host. The outputs from the centroids for each host were overlaid in the ordination space ('envfit' function), with their standard deviational ellipses ('ordiellipse' function).

Four, to identify whether compositional differences were also related with soil environmental parameters (pH, bulk density, total N, $\delta^{15}N$, total available N, ammonium, nitrate, dissolved organic nitrogen (DON), total P, available P, total organic carbon, total carbon, C/N ratio) all non-correlated variables were fitted onto the overall NMDS ordination plot using the 'envfit' function and 999 permutations. In addition, the significant soil parameter response surface models were fitted over NMDS ordination results by general additive models (GAM) using 'ordisurf' function to identify the linearity of the trends.

Five, we did not rarefy the fungal community due to the potential information loss. Instead, we included square-root transformed read counts as an explaining variable (Bálint et al., 2015), to account for variation in sequencing depth. LME models were used to test significant changes in diversity between host and two soil layers. In the overall analysis, plot identity was defined as a random factor while host was defined as fixed factors. Finally, indicator species analysis was used to identify the species related with each host using indcspecies package (De Cáceres et al., 2010).

3 Results

3.1 Correlation of fungal communities and tree host species

The host species identity influenced species soil fungal composition significantly (PMAV all data: 999 permutations; $P < 0.001$), and accounted for 46% of the variance in the species data.. By splitting the communities in two depths, we observed the same significant patterns in each layer independently (PMAV Top and Bottom data: 999 permutations; $P < 0.001$). The multiple comparisons showed the existence of significant differences between each host soil composition. Splitting the communities in two depths, we observed the same significant patterns in each layer independently (PMAV Top and Bottom data: 999 permutations; $P <$

0.001). In both cases, the host accounted for 53 and 54% of the compositional variance of the data. Based on these results we decided to analyse the both layers together.

The nmds ordination (stress 0.12) for the first two axes showed clearly that the HOST fit on the ordination was significant ($P < 0.001$). The sites plot (Fig. 1) showed that the overall distribution of sites reflected the Host differences. The Host SD-ellipses (Fig. 1) indicated that the four Host were located in different regions of the ordination space. The *Fagus crenata* plots were located on the central right side of the ordination, the *Robinia pseudoacacia* plots occupied the lower left hand quadrant; the *Cryptomeria japonica* plots were located in the upper left hand quadrant; while *Larix kaempferi* was located at lower central position.

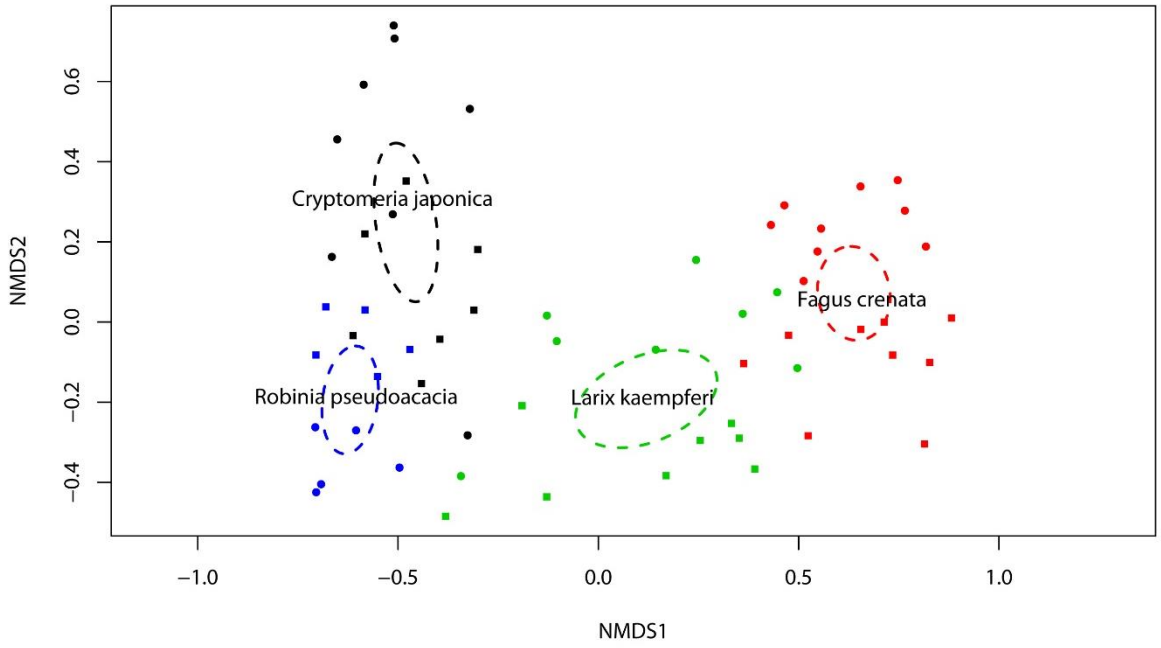


Figure 1: The nmds ordination (stress 0.12) for the two axes showed clearly that the host fit on the ordination was significant ($P < 0.001$). The plot showed that the overall distribution of sites reflected the host differences. The host SD-ellipses indicated that the four host were located in different regions of the ordination space.

3.2 Correlation of fungal communities and soil parameters

The soil parameters are taken from Seidel et al. (2019a, b, c) (Table 1).

From the set of soil environmental parameters the fit onto species ordination space was significant ($P < 0.05$; Fig.2a) only for 3 variables named: nitrate, CN and pH, explaining a

great proportion of the variance (35%). The remainder of variables tested explained an insignificant proportion of the variance.

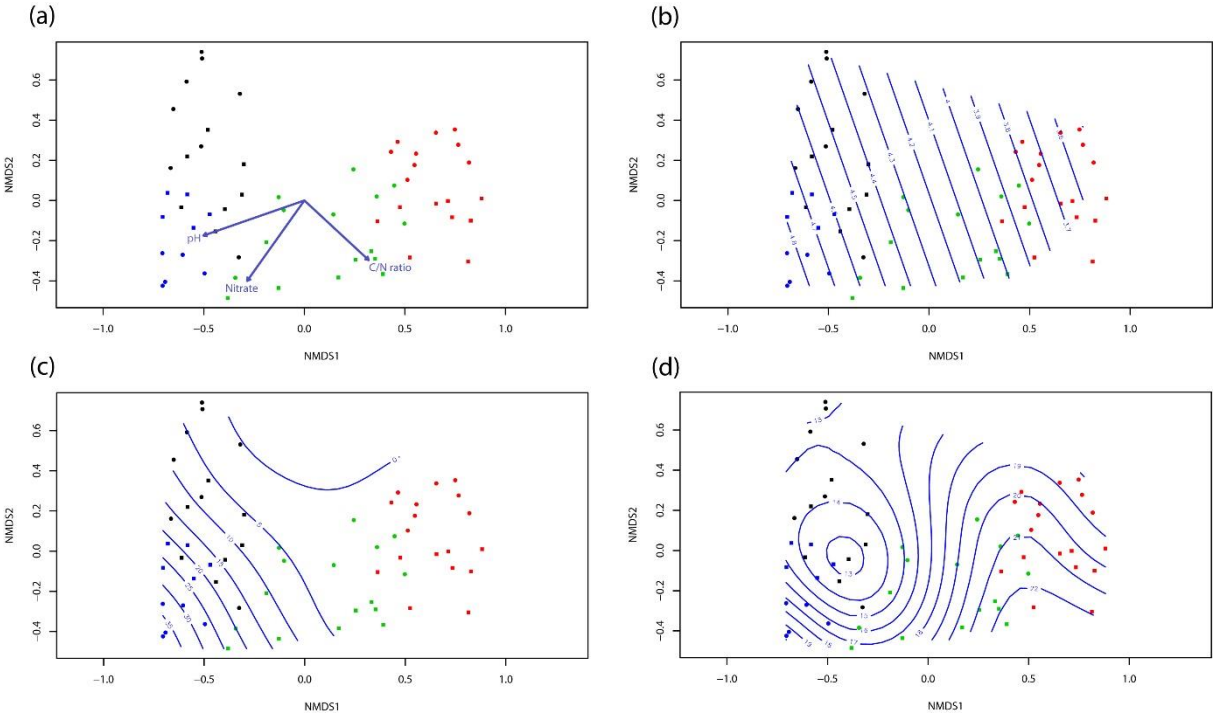


Figure 2: Correlation of fungal community structure with soil parameters **(a)**, pH **(b)**, nitrate content **(c)** and C/N ratio **(d)**.

The pH increased significantly towards the lower left hand of the ordination, showing that *Fagus crenata* plots are located around a pH of 3.6 and moving toward values of 4.6-4.8 at *Robinia pseudoacacia* and *Cryptomeria japonica* plots (Fig. 2b). Surprisingly, the nitrate isolines showed a nonlinear pattern increasing from the lower left side to the upper side were the nitrate values are around 0. It is interesting to mention that *Fagus crenata* plots were covered by Nitrate fit, suggesting low values (Fig. 2c). The C/N fit showed a nonlinear trend with greatest values in *Larix kaempferi* and *Fagus crenata* plots (around 21) and lower values in *Cryptomeria japonica* and *Robinia pseudoacacia* plots (around 14) (Fig. 2d). There were significant differences in soil fungi richness between hosts ($F_{[3,17]}$=5.62, P =0.010). The differences were clearly produced by *Fagus crenata* plots that showed a significantly lower richness (107 ± 15) than the other hosts, sharing similar richness values (*Larix kaempferi*: 148 ± 14, *Robinia pseudoacacia*: 119 ± 8, *Cryptomeria japonica*: 132 ± 12 (Fig. 3a). There was no significant difference in richness with soil depth. Further, there were no differences in species evenness suggesting that fungal composition was evenly distributed between hosts.

At the same time, values were higher than 0.9 for all hosts indicating that there was no species dominance among the fungal communities (Fig. 3b). However, the top soil layer showed significantly greater evenness ($P < 0.01$) than the bottom, but in both cases, the values are higher (Fig. 3c). When considering soil fungi communities' β-diversity, our results demonstrated significant differences in soil communities' β-diversity between hosts (Fig. 4). *Larix kaempferi* and *Cryptomeria japonica* showed a higher β-diversity than *Robinia pseudoacacia* and *Fagus crenata*. These results indicated that the soil communities are more similar in *Robinia pseudoacacia* and *Fagus crenata* plots compared with *Cryptomeria japonica* and *Larix kaempferi* plots, which have more heterogeneity in species composition. Finally, indicator species analysis showed that seven species were related with *Fagus crenata* and *Larix kaempferi* plots, while the *Robinia pseudoacacia* plots showed the highest number of indicator species (14). In contrast, a native species like *Cryptomeria japonica* only showed four species associated.

Table 1: Soil characteristics

Species	Depth	pH	Bulk density [g cm^{-1}]	Total N^1 [mg kg^{-1}]	$\delta^{15}N$ (‰)	Total N_{bound} [mg kg^{-1}]	Ammonium [mg kg^{-1}]	Nitrate [mg kg^{-1}]	DON [mg kg^{-1}]	total P [mg kg^{-1}]	available P [mg kg^{-1}]	Total org.C [mg kg^{-1}]	Total C [mg kg^{-1}]	Total N^2 [mg kg^{-1}]	C/N ratio
Cryptomeria japonica	Top	4.5 ± 0.2	0.5 ± 0.2	8057 ± 3611	0.8 ± 1.3	407 ± 100	140 ± 126	20 ± 19	252 ± 78	1988 ± 1106	35 ± 13	407 ± 100	76991 ± 43635	5591 ± 2454	13.8 ± 4.2
	Bottom	4.6 ± 0.1	**0.8 ± 0.1***	**2877 ± 1600****	**2.9 ± 1.2****	**208 ± 38****	**35 ± 42****	**11 ± 12****	163 ± 56	3823 ± 2060	22 ± 15	**208 ± 38***	48006 ± 28941	**3276 ± 1708***	14.2 ± 2.7
Larix kaempferi	Top	4.2 ± 0.6	0.8 ± 0.1	3501 ± 2644	0.9 ± 1.5	138 ± 52	8 ± 3	1 ± 1	129 ± 49	3599 ± 2145	16 ± 8	174 ± 91	43022 ± 13532	2249 ± 397	19.3 ± 5.8
	Bottom	4.4 ± 0.4	**1.0 ± 0.1***	**1442 ± 908***	2.0 ± 1.5	88 ± 8	5 ± 1	1 ± 1	82 ± 8	4659 ± 1460	10 ± 5	106 ± 30	**20978 ± 11931****	**1160 ± 743****	18.3 ± 3.1
Fagus crenata	Top	3.6 ± 0.1	0.3 ± 0.1	9975 ± 4351	0.7 ± 1.7	295 ± 78	251 ± 57	4 ± 6	39 ± 38	937 ± 311	36 ± 15	500 ± 213	176922 ± 52017	8527 ± 1975	20.5 ± 2.2
	Bottom	3.7 ± 0.3	**0.6 ± 0.1****	**4268 ± 1486****	**2.9 ± 1.8***	**173 ± 33****	**100 ± 74***	3 ± 5	70 ± 82	1859 ± 979	**21 ± 9***	318 ± 44	**50693 ± 24241****	**2513 ± 1271****	20.2 ± 3.0
Robinia pseudoacacia	Top	4.7 ± 1.4	0.3 ± 0.1	6063 ± 2290	0.5 ± 0.9	143 ± 10	12 ± 2	48 ± 4	83 ± 4	1794 ± 697	34 ± 4	145 ± 6	162275 ± 14806	8098 ± 943	20.1 ± 0.9
	Bottom	4.6 ± 1.0	**0.9 ± 0.1****	**2877 ± 1600***	**2.2 ± 0.6****	**100 ± 4****	5 ± 1	18 ± 1	77 ± 5	3320 ± 1465	**13 ± 1****	**64 ± 5****	**27366 ± 4549****	**2209 ± 620****	**12.7 ± 1.9***

[1]measured together with $\delta^{15}N$ [2]measured together with total C

*** P < 0.05** **** = P< 0.01**

All values taken from Seidel et al. (2019a, b, c)

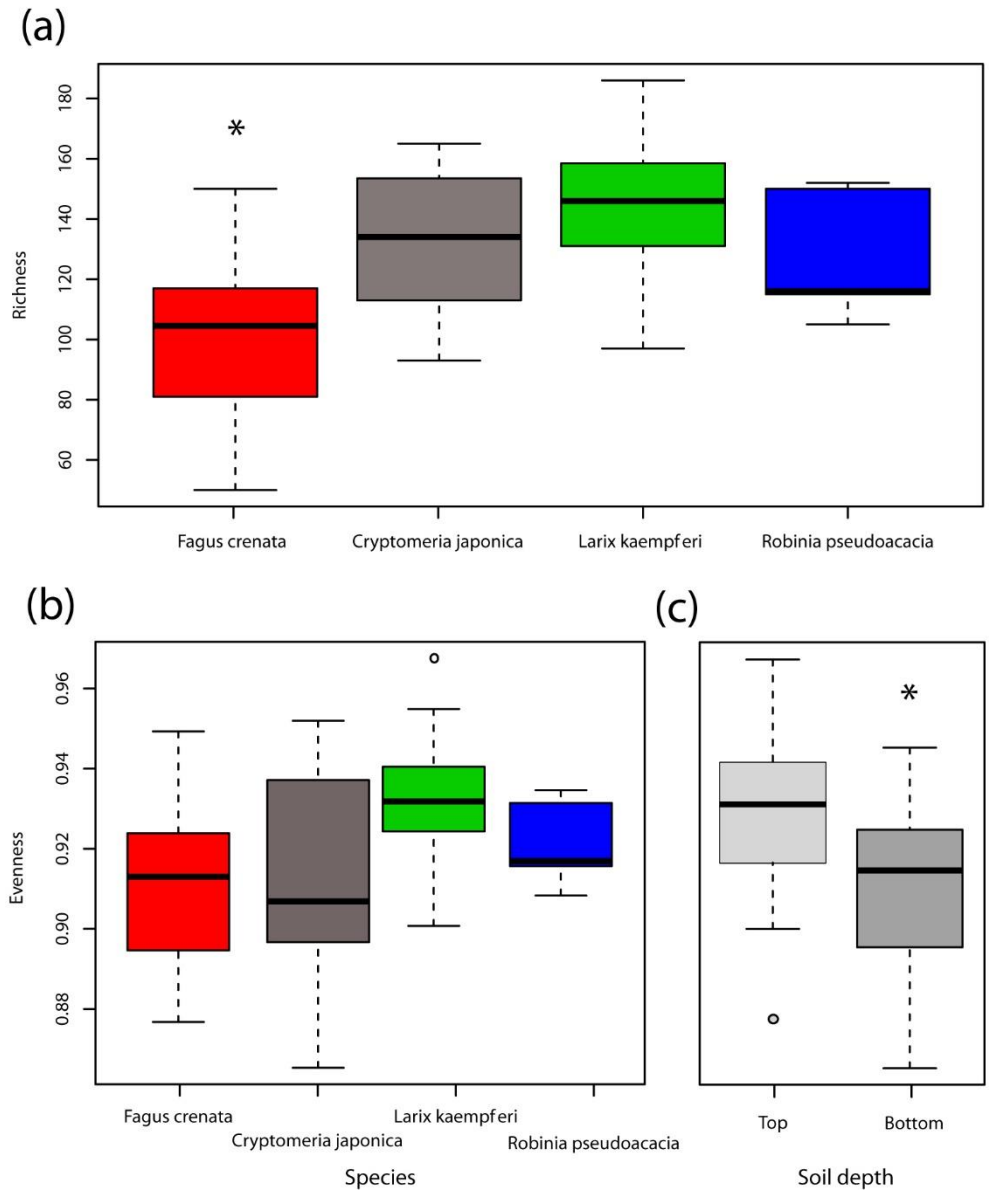


Figure 3: Differences in fungal species richness **(a)**, evenness **(b)** and evenness with soil depth **(c)** among host species. There was no significant difference in richness with soil depth. Asterisks indicate significant differences ($P < 0.01$).

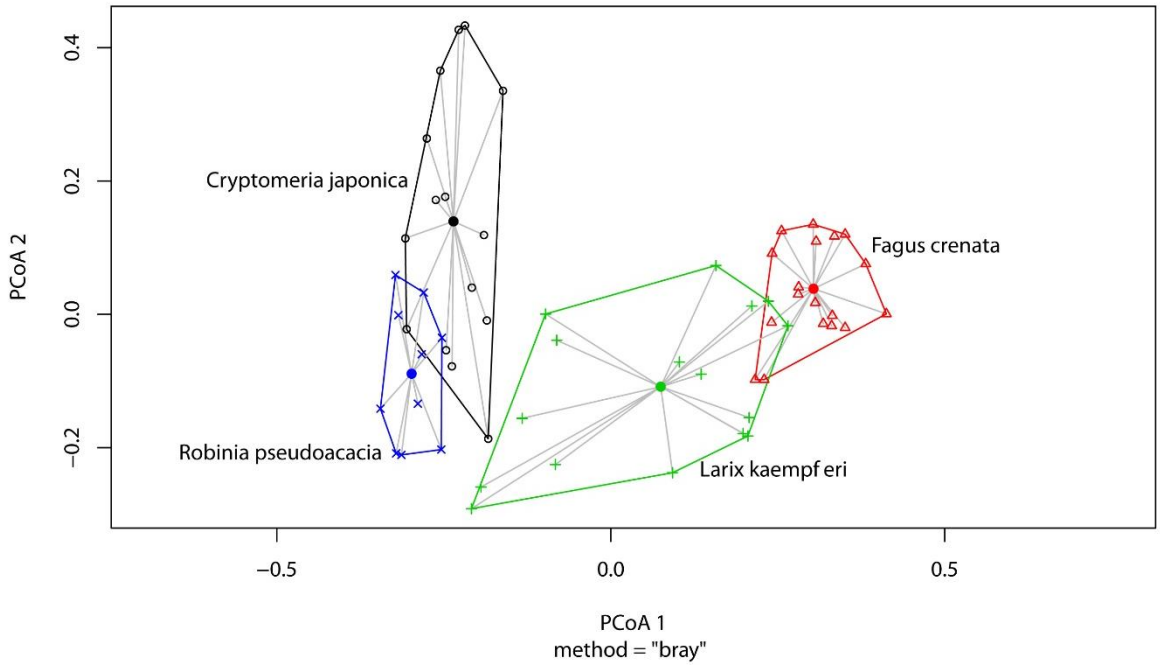


Figure 4: Fungal β-diversity of the host species indicating that the soil community composition is more similar between *Robinia pseudoacacia* and *Fagus crenata* plots compared with *Cryptomeria japonica* and *Larix kaempferi*.

3.3 Soil fungal communities' species composition

A total of 690,248 out of 1,526,648 sequences (45%) passed quality filtering. Single-linkage clustering resulted in 3111 OTUs, of which 550 (93% of the high-quality sequences) were assessed for identification to species level and functional guild. We obtained an average of 10,774 reads per sample. This corresponded to 172,562 reads per plot (69,024 reads per site). Overall, Ascomycota dominated the fungal community (57% of the identified sequences), followed by Basidiomycota (22%) and six other fungal divisions forming 5% while 16% remained unknown.

The ascomycota was represented by Letiomycetes (31%), Sordariomycetes (17 %), Eruotiomycetes (10%) and Dothiedomycetes (8%). 12% of remaining reads were represented by up of 18 different classes while 22% remained unknown. Among Basidiomycota, 86% belonged to Agaricomycetes and 7% to seven other classes while another 7% remained unknown.

For *Cryptomeria japonica*, we found that saprotrophs dominated the community (23 ± 1 %), followed by moulds (10 ± 1 %), root associated fungi (10 ± 3 %) and Plant pathogens (4 ± 5 %)

forming the majority of identified Fungi species (Fig. 5). With depth, Plant pathogens increased ($P < 0.01$). The functional guilds of *Larix kaempferi* consisted mainly of EMF (25 ± 8 %), Saprotrophs (21 ± 4 %), and moulds (12 ± 2 %). Plant pathogens (1 ± <1 %) decreased significantly ($P < 0.05$). Functional guilds of *Fagus crenata* consisted predominantly of EMF (39 ± 8 %), followed by Saprotrophs (15 ± <1 %) and mould (14 ± <1 %). Only plant pathogens (1 ± <1 %) increased significantly ($P < 0.05$) with soil depth. The functional guilds of *Robinia pseudoacacia* were made of Saprotrophs (42 ± 9 %), mould (13 ± 1 %), and root associated fungi (8 ± 8 %) and plant pathogens (4 ± 1 %). Root associated fungi increased ($P < 0.05$) with soil depth. Reads for taxa with unknown or non-determined function accounted for 51 ± 4 % for *Cryptomeria japonica*, 32 ± 3 % for *Larix kaempferi*, 30 ± 7 % for *Fagus crenata* and 25 ± 2 % for *Robinia pseudoacacia*.

Fagus crenata and *Larix kaempferi* showed the greatest abundance of EMF ($P< 0.01$) followed by R*obinia pseudoacacia* with a small amount of EMF ($P< 0.01$), while EMF were effectively absent in *Cryptomeria japonica*. The most abundant mycorrhizal genera were belonging to the genera Russula and to the family Telephoraceae. Most Saprotrophs were found in *Robinia pseudoacacia* ($P< 0.01$) followed by *Cryptomeria japonica* and *Larix kaempferi*, while *Fagus crenata* showed the lowest abundance ($P< 0.05$). *Cryptomeria japonica* showed the highest abundance in root-associated fungi while *Fagus crenata* showed the lowest ($P < 0.01$).

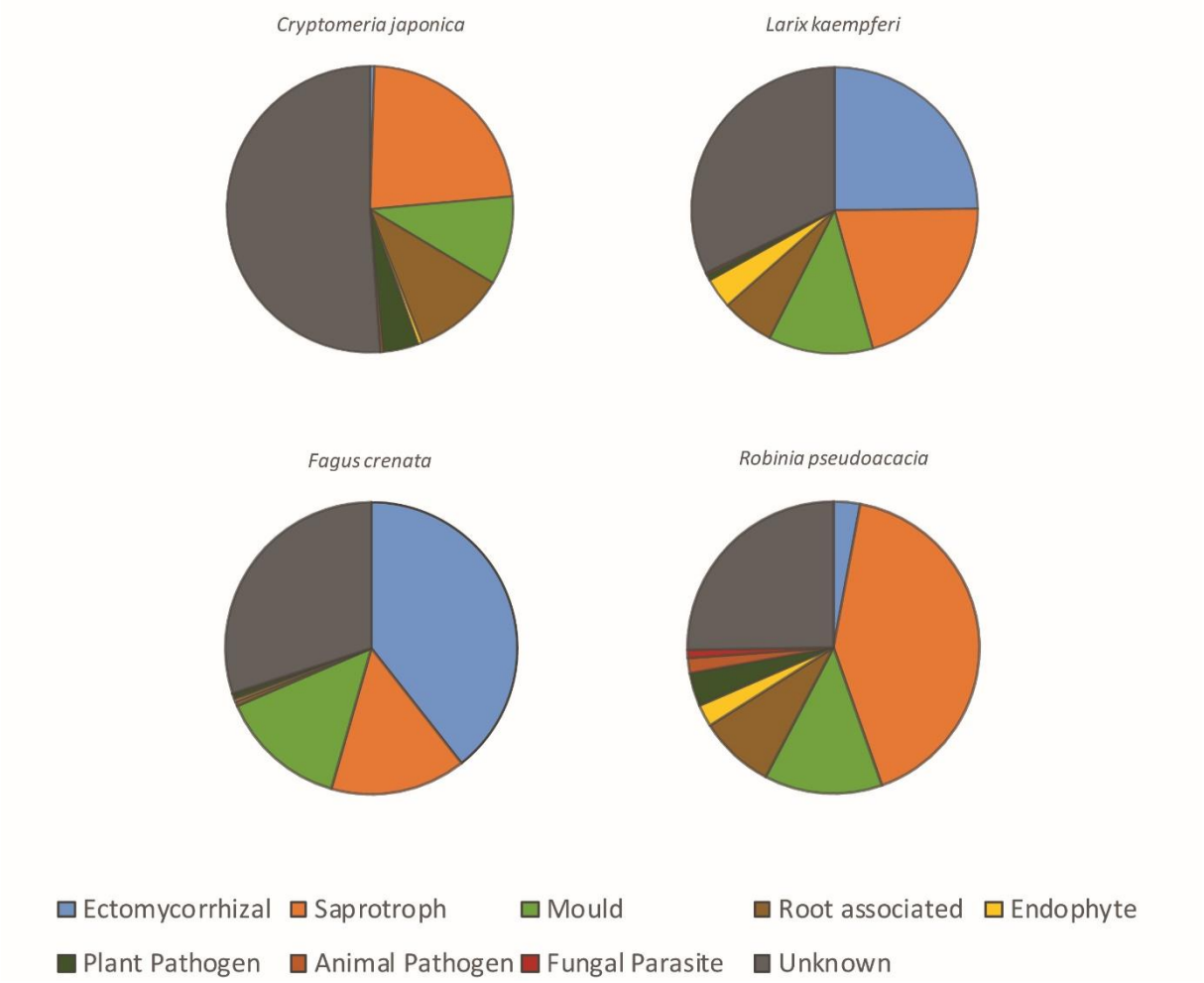


Figure 5: Fungal community structure among host species.

4 Discussion

4.1 Soil fungal communities' species composition between hosts

The fungal community was dominated by Ascomycetes, most of them being decomposers of organic material, and to a less extension forming ectomycorrhizal associations with plants thus playing an important role in nutrient cycling (Baldrian et al., 2011; Sardans et al., 2013). *Larix kaempferi* and *Fagus crenata* showed a high abundance of EMF showing their reliance on these fungi for nutrient uptake (Hobbie & Agerer, 2010; Wallenda & Kottke, 1998) while *Robinia pseudoacacia* and *Cryptomeria japonica* were not, as also found by other studies for these species (Zhou & Hogetsu, 2002; Taniguchi et al, 2006; Lang et al, 2013; Chiwa et al., 2015). This was supported by the abundance of root-associated fungi, being high in *Cryptomeria japonica* and low in *Fagus crenata*. Further, EMF were found in the *Robinia*

pseudoacacia, formerly covered by *Cryptomeria japonica* which is known to have arbuscular mycorrhiza. This suggested that the fungal communities changed possibly due to clear cutting and the change of host tree species within 7 years of colonization (Hartmann et al., 2012; Lin et al., 2016; Kyaschenko et al., 2017; Castaño et al., 2018). Further, differences found in Saprotrophs abundance seemed be host dependent suggesting higher rates of nutrient turnover in *Robinia pseudoacacia* plots due to the high abundance in Saprotrophs. However, this could also be linked to differences in understory vegetation composition and not to the tree species of the plots as found in mixed-boreal forests (DeBellis et al. 2007). Changes of soil fungal communities with depth were found for EMF, plant pathogens and root associated fungi, however especially changes of EMF in *Larix kaempferi*, *Fagus crenata* and *Robinia pseudoacacia* remained insignificant. Differences in fungal composition with depth have been observed to be very clear when including the litter layer (Jumpponen et al., 2010; Andreetta et al., 2011; Voříšková et al., 2014), however in our study the litter layer was very thin in all sites indicating efficient degradation of fresh litter, causing a more evenly distributed nutrient pattern with depth than in areas with thicker litter layers (Fierer et al., 2003; Heat etl al., 2005). Thus, fungal composition was affected by this distribution and showed only trends but rarely significant changes with depth. This could be confirmed with a more extensive sampling to clarify distribution patterns with depth. While EMF, Saprotrophs and moulds represented most of the fungal communities for all tree species, many taxa that remained unknown, especially in *Cryptomeria japonica* plots, which stresses the importance of future studies aiming at identifying these taxa. In this study, 22% of Ascomycota remained unknown accounting for 12% of all fungi found in this study. Therefore, identifying such taxa may facilitate the understanding of soil processes and nutrient dynamics occurring in these ecosystems. *Cryptomeria japonica* is commercially the most important tree species in Japan (Seidel et al, 2019a) and a better understanding of the soil fungi associated with it may enable us to improve this tree species productivity. Additionally, further research may reveal new substances, as ascomycetes already brought important benefits for humanity (e.g. medical substances) (Marx, 2004; Johnson, 2013).

It is worth mentioning that although they belong to different functional groups, all tree species showed an even distribution of fungal species without domination of certain fungal communities independent of soil depth with *Fagus crenata* having the lowest fungal species richness. *Fagus crenata* is an exceptional tree, as it often acted differently from the other

species e.g. changes in N isotopic composition, free amino acid composition (Seidel et al., 2019a), water uptake patterns (data unpublished) and fungal community species richness. When looking at β-diversity, describing the dissimilarity in species composition among different sites (Whittaker, 1960), we could see that *Fagus crenata* and *Robinia pseudoacacia* were associated with more homogeneous fungal communities than the other tree species. In our study, this could be attributed to the differences in pH, nitrate and C/N ratio, as β-diversity has been shown to be mainly affected by environmental factors (i.e., climate, soil pH and nutrients) on a local scale (Wu et al., 2013; Prober et al., 2014; Chen et al., 2017) but also by fungal species turnover (rather than nestedness) (Wang et al., 2017). This implies that the fungi associated with these tree species are more specialized and thus may be more affected by increased N deposition than tree species with a more divers fungal community (Wallenda & Kottke, 1998) making these communities more vulnerable in times of increasing atmospheric N deposition (Lopez et al., 2010; Fukushima et al., 2011; Peri et al., 2012). Further, we found that *Robinia pseudoacacia* was associated with the highest number of indicator species (14), followed by *Fagus crenata* (7) and *Larix kaempferi* (7), while *Cryptomeria japonica* had the lowest (4). To our knowledge, this is the first study to examine fungal communities' structure within the same research area among different hosts, as other studies exclusively focused on EMF (Ishida et al., 2007).

The soil fungi sampling being conducted exclusively in May limited the study, as it did not allow us to observe seasonal changes in soil fungal composition as found in other studies for boreal and temperate forest ecosystems (Wallander et al., 2001; Jumpponen et al., 2010; Andreetta et al., 2011; Voříšková et al., 2014; Lin et al., 2016). They are showing a higher relative abundance of EMF species and total fungal biomass during summer and autumn and free-living fungi, such as yeasts, litter saprotrophs or moulds were found to increase under wetter conditions from an intra-annual perspective or under snow cover (Santalahti et al., 2016). However, Castaño et al. (2018b) indicated for Mediterranean forest ecosystems, that spatial variation at a local scale (< 3 km) might be larger than seasonal variability as higher temperatures and water availability may stimulate nutrient cycling and plant production. Seasonal measurements of soil fungal communities in the tree species of this study would elucidate this issue, as our plots were in an extremely humid environment with heavy snow cover.

4.2 Analysis of soil fungal communities and soil nutritional status

Soil fungal community structure was strongly influenced by pH as fungal growth is accelerated with decreasing pH, while increasing pH favours bacterial growth making *Larix kaempferi* and *Fagus crenata* possibly more reliant on fungal communities for nutrient turnover than *Robinia pseudoacacia* and *Cryptomeria japonica* (Blagodatskaya & Anderson, 1998; Rousk et al., 2009,). However, there is a narrow pH range between 4 and 4.5 where fungi growth peaks and rapidly reduces with pH levels below 4. Nevertheless, fungal growth rates are significantly higher, as bacterial growth nearly ceases at pH levels below 4, elevating the relative importance of fungi (Rousk et al., 2009, 2010). This phenomenon could also explain the lower species richness of *Fagus crenata*, which exhibited the lowest pH values around 3.7, as the pH may be lower than optimal growth ranges of certain fungi (Rousk et al., 2011).

Another significant correlation between soil fungal community structure and soil nitrate content was found. Increases in soil N, especially in nitrate, causes microbial groups to be supressed in activity and abundance (DeForest et al., 2004) while fungal growth is stimulated (Rousk & Bååth, 2007). This indicated that in *Robinia pseudoacacia* plots, microbial activity could be high due to the higher pH in comparison to the other species; however, the high nitrate concentrations found might dampen this effect making fungal communities relatively more important. In contrast, nitrate content was low in *Cryptomeria japonica* plots, but pH high, which may cause microbial activity to be higher in these plots reducing the relative importance of soil fungi for this tree species. An analysis of these two species plots could elucidate the effect of pH and nitrate content on microbial activity and thus fungal community importance. In general, the microbial dynamics and ecosystem functioning among tree species seem to differ significantly as a result of pH and nitrate distribution, as microbial nitrogen demands can be met at relatively low levels of nitrate. This suggests that even minor differences in nitrogen may cause significant shifts in microbial and fungal dynamics and ecosystem functioning (Ferreira et al., 2006) between the four species of this study.

Soil fungal community structure correlated with the soil C/N gradient among tree species where *Larix kaempferi* and *Fagus crenata* showed higher C/N ratios than *Cryptomeria japonica* and *Robinia pseudoacacia*. The soil communities of *Larix kaempferi* and *Fagus crenata* were characterized by a high abundance in EMF suggesting they were mining for N,

while lower abundances in Saprotrophs than in *Cryptomeria japonica* and *Robinia pseudoacacia* were found. This may partially be linked to the litter quality and litter quantity of these trees. Shifts in substrate quality (high vs low C:N) and availability (nitrogen fertilization) have previously been linked to shifts in fungal abundance (Frey et al., 2004; Allison et al., 2007). Further, it has been shown in agroecosystems, that the two main groups of decomposers, bacteria and fungi, have different efficiencies of sequestering C on the soil with fungi being more efficient (Six et al., 2006) as fungi require less N for biomass accumulation in addition to a higher growth efficiency (Rousk & Bååth, 2007). This would make plots with a higher abundance of fungi in comparison to bacteria more efficient in C sequestration suggesting that due to the optimal pH and the high nitrate content allowing high fungi abundance, *Robinia pseudoacacia* plots would be the most efficient soils for C-sequestration followed by *Cryptomeria japonica*, while *Fagus crenata* plots would be least efficient. Fontaine et al. (2010) found the existence of a bank mechanism that regulates nutrient and carbon sequestration in soil. Soil microbes can decompose old recalcitrant soil organic matter (SOM) by using fresh carbon as a source of energy, which is called the priming effect (PE). PE is low when nutrient availability is high, allowing sequestration of nutrients and carbon; in contrast, microbes release nutrients from SOM when nutrient availability is low. In our plots, nutrient availability was highest in *Cryptomeria japonica* and *Fagus crenata* plots suggesting that *Cryptomeria japonica* plots may be more efficient in C-sequestration than *Robinia pseudoacacia* plots as well as *Fagus crenata* plots may be more efficient than *Larix kaempferi* plots. C content was highest in *Fagus crenata*, followed by *Robinia pseudoacac*ia and *Cryptomeria japonica.* Deeper soil layers of the *Robinia pseudoacacia* plot were poorer in C content indicating that C sequestration might enhance due to *Robinia pseudoacacia* possibly causing higher C sequestration in time. However, at the time of sampling, *Fagus crenata* and *Cryptomeria japonica* are more efficiently sequestering C. Measurements of fungal abundance are needed to quantify the effect of pH, nitrate content and C/N ratio on soil fungal abundance in order to expose which forest type creates soils more efficient in C sequestration. Further, measurements in plots with adult *Robinia pseudoacacia* are necessary in order to assess the long-time effect of *Robinia pseudoacacia*s nutrient rich litter possibly making its soils more effective C sinks than *Cryptomeria japonica* plots. This would allow us to improve forest management practices towards increased C sequestration and in turn combat climate change.

5 Conclusion

To our knowledge, this is the first study to examine fungal communities' structure within the same research area among different hosts of four typical Japanese tree species of the cold-temperate zone. Based on the results obtained in this study, we concluded that fungal communities were highly dependent on tree species and controlled significantly (34%) by three factors in the soil: pH value, nitrate content and C/N ratio while soil depth only influenced the evenness of the soil fungal community. *Fagus crenata* showed a high degree of specialization in a small number of fungi, followed by *Robinia pseudoacacia*. Fungal communities of *Cryptomeria japonica* and *Larix kaempferi* were more divers making these tree species less vulnerable to changes in environmental conditions. Furthermore, *Robinia pseudoacacia* and *Cryptomeria japonica* plots seemed to be more efficiently sequestering C than *Fagus crenata* and *Larix kaempferi* plots. As *Robinia pseudoacacia* is an invasive tree species strongly reducing biodiversity, we suggest favouring the planting of *Cryptomeria japonica* over *Fagus crenata* and *Larix kaempferi* in order to reduce atmospheric C by binding C more efficiently in forest soils. The next steps are to identify the large number of unknown fungal species found in this study, especially for *Cryptomeria japonica* and further, analysing interactions between mycorrhizal fungi community structure with host plants internal nutrient status, in order to shed light on this symbiosis.

Acknowledgements

We want to thank the staff of the University Research Forest of Yamagata University for keeping the site accessible in summer and winter.

Author contributions

Felix Seidel and Carles Castaño. have done the conceptualization and methodology for this study. Felix Seidel and Carles Castaño conducted the investigation, formal analysis, data curation & visualization while Josu G. Alday conducted the statistical analysis. Felix Seidel, Carles Castaño and Josu G. Alday prepared the original draft. M. Larry Lopez C. and José Antonio Bonet were supervising this study and contributed to data interpretation and reviewing of the manuscript.

Chapter 7

Conclusion

The aim of this work was to investigate the differences in N and P cycling of four typical tree species found in north eastern Japan, representative for three functional groups, at four phenological stages in order to improve management practices to promote their ecosystem functions in a world of climate change.

By linking the movement of free amino acids with N stable isotope measurements, we might have found a powerful tool to track intra plant N movement and transformation more reliably in trees species that show significant variation in isotopic composition in plant tissues among the phenological stages. The next step is to analyse compound specific N isotopic composition of free amino acids especially in *Fagus crenata* to confirm the link between free amino acids and N isotope ratio found of this study. This would enable us to improve fertilizer composition of this widely distributed tree species to promote its growth.

Among the studied species, we found that *Cryptomeria japonica* seemed to be a rather nutrient efficient tree that may be the most resilient tree species towards nutrient availability changes, especially, if anthropogenic atmospheric deposition of N will increase as a result of human activities. In forests prone to N saturation, it would be advisable to promote the planting of *Cryptomeria japonica* as it has a high P use efficiency and is highly N and P proficient. Further, in forests not prone to N saturation, application of N fertilizer in autumn might be beneficial to increase N storage in roots and sapwood to support shoot growth in the following spring subsequently increasing C sequestration. Future studies should identify the large number of fungal communities associated with *Cryptomeria japonica* that remained unknown in order to understand their function.

Larix kaempferi was the least N and P demanding tree species. Similar to *Cryptomeria japonica*, this species was recycling N & P efficiently while it was more effective in recycling P. In forests, characterized by very poor N and P availability, *Larix kaempferi* can thrive and as it is a fast growing species like *Cryptomeria japonica*, planting of this tree species could increase C fixation better than the other species of this study. Application of N fertilizer should be conducted in spring to promote shoot growth, while application of P fertilizer in autumn may increase nutrient storage of this species. However, it seems unnecessary as it grows well in low nutrient environments with the support of ectomycorrhizal fungi.

Fagus crenata was N and P demanding, but these nutrients were efficiently recycled. Due to high litter production, *Fagus crenata* seemed to support soil microbial processes and soil development better than the coniferous species. Higher atmospheric N deposition or N fertilization during the shoot growth stage in *Fagus crenata* forests may initially lead to enhanced growth, photosynthetic activity and P mineralization. However, these forests might become P limited more rapidly than the other species, as this species is very nutrient demanding and on the edge between N and P co-limitation during the growing season. Further, this tree species fungal community was highly specialized and homogeneous with an abundance of ectomycorrhizal fungi possibly making it more vulnerable than the other tree species towards changes in nutrient supply, which will affect the fungal community structure.

Robinia pseudoacacia was the only leguminous species studied and thus, independent from soil N uptake. This was displayed with its high N and P content of all plant tissues but the lowest N and P efficiency and proficiency. This will lead to a fertilization effect of the soil, as this species litter is nutrient rich. Its fungal community was homogeneous and the high abundance of saprotrophs suggested higher nutrient turnover in this plot in comparison to the other tree species. In order to promote its growth, N fertilizer applied in spring to support shoot growth and P fertilizer applied in autumn to increase P storage could be beneficial, as throughout the season, this species shifts from N to P limitation. If atmospheric N increases, *Robinia pseudoacacias* growth and photosynthetic activity may increase during the shoot growth and green leaf stage but the timing of P limitation along the seasons might shift to an earlier phenological stage. Additionally, this species is most suitable to increase soil fertility in a relatively short time and planting it in combination with other species will be beneficial for increased C fixation in woody plant tissues caused by increased growth and lead to improved soil development stimulated through amplified litter production. However, as this tree species is invasive and significantly reduces biodiversity, we do not recommend planting and fertilization of this species, especially in unmanaged forests.

In the future, conducting similar studies on other globally important tree species would enable us to improve forest management practices towards increased C sequestration in a world of climate change.

References:

Abarenkov K, Nilsson RH, Larsson K-H, Alexander IJ, Eberhardt U, Erland S, Høiland K, Kjøller R, Larsson E, Pennanen T, Sen R Taylor AFS, Tedersoo L, Ursing BM, Vrålstad T, Liimatainen K, Peintner U, Kõljalg U. 2010. The UNITE database formolecular identification offungi – recent updates andfuture perspectives. *New Phytologist* **186**: 281-285.

Aerts R, Chapin FS. 2000. The mineral nutrition of wild plants revisited: a re-evaluation of processes and patterns. *Advances in Ecological Research* **30**: 1-67.

Allison SD, Hanson CA, Treseder KK. 2007. Nitrogen fertilization reduces diversity and alters community structure of active fungi in boreal ecosystems. *Soil Biology and Biochemistry* **39**: 1878–1887.

Amundson R, Austin AT, Schuur EAG, Yoo K, Matzek V, Kendall C, Uebersax A, Brenner D, Baisden WT. 2003. Global patterns of the isotopic composition of soil and plant nitrogen. *Global Biochemical Cycles* **17**, Vol. 1: 1031.

An G-H, Miyakawa S, Kawahara A, Osaki M, Ezawa T. 2008. Community structure of arbuscular mycorrhizal fungi associated with pioneer grass species *Miscanthus sinensis* in acid sulfate soils: Habitat segregation along pH gradients. *Soil Science and Plant Nutrition* **54**: 517–528.

Andreetta A, Macci C, Ceccherini MT, Cecchini G, Masciandaro G, Pietramellara G, Carnicelli S. 2011. Microbial dynamics in Mediterranean Moder humus. *Biology and Fertility of Soils* **48**: 259–270.

Baldrian, P., Voříšková, J., Dobiášová, P., Merhautová, V., Lisá, L., Valášková, V., 2011. Production of extracellular enzymes and degradation of biopolymers by saprotrophic microfungi from the upper layers of forest soil. *Plant Soil* **338:** 111–125.

Bálint M, Bartha L, O'hara RB, Olson MS, Otte J, Pfenninger M, Robertson AL, Tiffin P, Schmitt I. 2015. Relocation, high-latitude warming and host genetic identity shape the foliar fungal microbiome of poplars. *Molecular Ecology* **24**: 235-248.

Bencúr P, Seinkellner H, Svoboda B, Mucha J, Strasser R, Kolarich D, Hann S, Köllensperger G, Gössl J, Altmann F, Mach L. 2005. Arabidopsis thaliana *β*1,2-xylosyltransferase: an unusual glycosyltransferase with the potential to act at multiple stages of the plant N-glycosylation pathway. *Biochem. J.* **388**: 515-525.

Bi G, Scagel CF, Cheng L, Fuchigami LH. 2004. Soil and foliar nitrogen supply affects the composition of nitrogen and carbohydrates in young almond trees. *Journal of Horticultural Science & Biotechnology* **79**: 175-181.

Bika R, Bhandari N, Khanal A. 2018. Response of Different Doses of Nitrogen on Broccoli (*Brassica oleracea* var*. italic*) in Lamjung District. *Int. J. Appl. Sci. Biotechnol.* **6** (3): 270-273.

Blagodatskaya EV & Anderson T-H. 1998. Interactive effects of pH and substrate quality on the fungal-to-bacterial ratio and qCO_2 of microbial communities in forest soils. *Soil Biology and Biochemistry* **30** (10-11): 1269-1274.

Blujdea VNB, Pilli R, Dutca I, Ciuvat L, Abrudan IV. 2012. Allometric biomass equations for young broadleaved trees in plantations in Romania. *Forest Ecology and Management* **264**: 172-184.

Bolan NS. 1991. A critical review on the role of mycorrhizal fungi in the uptake of phosphorus by plants. *Plant and Soil* **134**: 189-207.

Callesen I, Nilsson LO, Schmidt IK, Vesterdal L, Ambus P, Christiansen JR, Högberg P, Gundersen P. 2012. The natural abundance of ^{15}N in litter and soil profiles under six temperate tree species: N cycling depends on tree species traits and site fertility. *Plant Soil* **368**, Issue 1: 375-392.

Castaño C, Alday JG, Lindahl BD, Martínez de Aragón J, de-Miguel S, Colinas C, Parladé J, Pera J, Bonet JA. 2018a. Lack of thinning effects over inter-annual changes in soil fungal community and diversity in a Mediterranean pine forest. *Forest Ecology and Management* **424**: 420-427.

Castaño C, Lindahl BD, Alday JG, Hagenbo A, Martínez de Aragón J, Parladé J, Pera J, Bonet JA. 2018b. Soil microclimate changes affect soil fungal communities in a Mediterranean pine forest. New Phytol. **220**: 1211–1221.

Cairney, J.W.G., 2012. Extramatrical mycelia of ectomycorrhizal fungi as moderators of carbon dynamics in forest soil. *Soil Biol. Biochem*. **47:** 198–208.

Chapin FSIII, Johnson DA, McKendrick JD. 1980. Seasonal movement of nutrients in plants of differing growth form in an Alaskan tundra ecosystem: Implications for herbivory. *Journal of Ecology* **68**: 189-209.

Chapin FSIII, Kedrowski RA. 1983. Seasonal changes in Nitrogen and Phosphorus fractions and autumn retranslocation in Evergreen and Deciduous Taiga Trees. *Ecology* **64** (2): 376-391.

Chávez-Vergara BM, González-Rodríguez A, Etchevers JD, Oyama K, García-Olivia F. 2015. Foliar nutrient resorption constrains soil nutrient transformations under two native oak species in a temperate deciduous forest in Mexico. *Eur J Forest Res* **134**: 803-817.

Cherbuy B, Joffre R, Gillon D, Rambal S. 2001. Internal remobilization of carbohydrates, lipids, nitrogen and phosphorus in the Mediterranean evergreen oak *Quercus ilex*. *Tree Physiology* **21:** 9-17.

Chen L, Xu T, Veresoglou SD, Hu H, Hao Z, Hu Y, et al. 2017. Plant diversity represents the prevalent determinant of soil fungal community structure across temperate grasslands in northern China. *Soil Biol. Biochem.* **110**: 12–21.

Chenlemuge T, Hertel D, Dulamsuren C, Kishigjargal M, Leuschner C, Hauck M. 2013. Extremely low fine root biomass in *Larix sibirica* forests at the southern drought limit of the boreal forest. *Flora* **208**: 488-496.

Chikaraishi Y, Ogawa NO, Kashiyama Y, Takano Y, Suga H, Tomitani A, Miyashita H, Kitazato H, Ohkouchi N. 2009. Determination of aquatic food-web structure based on compound-specific nitrogen isotopic composition of amino acids. *Limnol. Oceanogr.: Methods* **7**: 740-750.

Chikaraishi Y, Ogawa NO, Doi H, Ohkouchi N, 2011. $^{15}N/^{14}N$ ratios of amino acids as a tool for studying terrestrial foodwebs: A case study of terrestrial insects (bees, wasps, and hornets). *Ecol. Res.* **26**: 835–844.

Chiou TJ, Lin SI. 2011. Signalling network in sensing phosphate availability in plants. *Annual Review of Plant Biology* **62**: 185–206.

Chiwa M, Saito T, Haga H, Kato H, Otsuki K, Onda Y. 2015. A Nitrogen-Saturated Plantation of *Cryptomeria japonica* and *Chamaecyparis obtusa* in Japan Is a Large Nonpoint Nitrogen Source. *Journal of Environmental Quality* **44** (4): 1225-1232.

Choi DS, Jin HO, Chung DJ, Sasa K, Koike T. 2008. Growth and physiological activity in *Larix kaempferi* seedlings. *Trees* **22**: 729-735.

inoculated with ectomycorrhizae as affected by soil acidification

Clemmensen KE, Bahr A, Ovaskainen O, Dahlberg A, Ekblad A, Wallander H, Stenlid J, Finlay RD, Wardle DA, Lindahl BD. 2013. Roots and Associated Fungi Drive Long-Term Carbon Sequestration in Boreal Forests. *Science* **339**: 1615-1618.

Clemmensen, K.E., Finlay, R.D., Dahlberg, A., Stenlid, J., Wardle, D.A., Lindahl, B.D., 2015. Carbon sequestration is related to mycorrhizal fungal community shifts during long-term succession in boreal forests. *New Phytol.* **205**: 1525–1536.

Conn C, Dighton J. 2000. Litter quality influences on decomposition, ectomycorrhizal community structure and mycorrhizal root surface acid phosphatase activity. *Soil Biology and Biochemistry* **32**: 489–496.

Couto-Vázquez A, González-Pietro SJ. 2010. Effects of climate, tree age, dominance and growth on $\delta^{15}N$ in young pinewoods. *Trees* **24**: 507-514.

Covelo F, Rodríguez A, Gallardo A. 2008. Spatial pattern and scale of leaf N and P resorption efficiency and proficiency in a *Quercus robur* population. *Plant Soil* **311**: 109-119.

Craine JM, Elmore AJ, Aidar, MPM, Bustamante M, Dawson TE, Hobbie EA, Kahmen A, Mack MC, McLauchlan KK, Michelsen A, Nardoto GB, Pardo, LH, Penuelas J, Reich PB, Schuur EAG, Stock WD, Templer PH, Virginia RA, Welker JM, Wright IJ. 2009. Global patterns of foliar nitrogen isotopes and their relationships with climate, mycorrhizal fungi, foliar nutrient concentrations, and nitrogen availability. *New Phytologist* **183:** 980-992.

Cooke, JEK, Weih M. 2005. Nitrogen storage and seasonal nitrogen cycling in *Populus*: bridging molecular physiology and ecophysiology. *New Phytologist* **167**: 19-30.

Crooke W M & Simpson W E. 1971. Determination of ammonium in Kjedahl digest of crops by an automated procedure. *J.Sci. Food Agric*. **22**: 9-10.

Dannenmann M, Bimüller C, Gschwendtner S, Leberecht M, Tejedor J, Bilela S, Gasche R, Haewinkel M, Baltensweiler A, Kögel-Knabner I, Polle A, Schloter M, Simon J, Rennenberg H. 2016. Climate change impairs nitrogen cycling in European beech forests. *Plos One* **11:** e0158823.

Dambrine E, Martin F, Carisey N, Granier A, Hällgren J-E, Sishop K. 1995. Xylem sap composition: a tool for investigating mineral uptake and cycling in adult spruce. *Plant and Soil* **168-169**: 233-241.

DeBellis, T, Kernaghan G, Widden P. 2007. Plant community influences on soil microfungal assemblages in boreal mixed-wood forests. *Mycologia* **99**: 356-367.

DeForest JL, Zak DR, Pregitzer KS, Burton AJ. 2004. Atmospheric Nitrate Deposition, Microbial Community Composition, and Enzyme Activity in Northern Hardwood Forests. *Soil Biology & Biochemistry* **68** (1): 132-138.

De Cáceres M, Legendre P, Moretti M. 2010. Improving indicator species analysis by combining groups of sites. *Oikos* **119**: 1674-1684.

De Groot CC; Marcelis L.F; Van Den Boogaard R; Kaiser WM; Lambers H. 2003. Interaction of nitrogen and phosphorus nutrition in determining growth. *Plant Soil* **248**: 257–268.

Di HJ, Condron LM, Frossard E. 1997. Isotope techniques to study phosphorus cycling in agricultural and forest soils: a review. *Biology and Fertility of Soils* **24** (1): 1-12.

Dighton J. 2003. Fungi in Ecosystem Process. Marcel Dekker Inc., New Jersey.

Duan K, Yi K, Dang L, Huang H, Wu W, Wu P. 2008. Characterization of a sub-family of Arabidopsis genes with the SPX domain reveals their diverse functions in plant tolerance to phosphorus starvation. *The Plant Journal* **54**: 965–975.

Dubey RS. 1999. Protein Synthesis by Plants Under Stressful Conditions. In: *Handbook of Plant and Crop Stress second Edition, Revised and Expanded.* New York, USA: Marcel Dekker, Inc., 365-397

Dyckmans J & Flessa H. 2001. Influence of tree internal N status on uptake and translocation of C and N in beech: a dual ^{13}C and ^{15}N labeling approach. *Tree Physiology* **21**: 395-401.

Edgar RC. 2011. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**: 2460–2461.

Elhani S, Fernández Lema B, Zeller B, Bréchet C, Guehl JM, Dupouey JL. 2003. Inter-annual mobility of nitrogen between beech rings: a labelling experiment. *Ann. For. Sci.* **60**: 503-508.

Enta A, Hayashi M, Lopez CML, Fujiyoshi L, Yamanaka T, Oikawa A, Seidel F. 2019. Nitrogen resorption and fractionation during leaf senescence in typical tree species in Japan. *Journal of Forestry Research* (in Press)

FAO 2006. Guidelines for soil description. 4th edition. FAO, ROME

Fang Y, Gundersen P, Mo J, Zhu W. 2009. Nitrogen leaching in response to increased nitrogen inputs in subtropical monsoon forests in southern China. *Forest Ecology and Management* **257**, Issue 1: 332-342.

Ferreira V, Gulis V, Graça MAS. 2006. Whole-stream nitrate addition aVects litter decomposition and associated fungi but not invertebrates. *Oecologia* **149**: 718-729.

Fierer N, Schimel JP, Holden PA. 2003. Variations in microbial community composition through two soil depth profiles. *Soil Biology & Biochemistry* **35**: 167-176.

Finzi AC & Berthrong ST. 2005. The uptake of amino acids by microbes and trees in three cold temperate forests. *Ecology* **86** (12): 3345-3353.

Fontaine S, Henault C, Aamor A, Bdioui N, Bloor JMG, Maire V, Mary B, Revaillot S, Maron PA. 2011. Fungi mediate long term sequestration of carbon and nitrogen in soil through their priming effect. *Soil Biology & Biochemistry* **43**: 86-96.

Food Agric. Organ. (FAO). 2010. Global forest resources assessment 2010. ***For. pap.* 163,** FAO, Rome.

Fotelli MN, Rennenberg H, Geßler A. 2002. Effects of Drought on the Competitive Interference of an Early Succsessional Species (*Rubus fruticosus*) on *Fagus sylvatica* L. Seedlings: ^{15}N Uptake and Partitioning, Responses of Amino Acids and other N Compounds. *Plant boil.* **4**: 311-320.

Frey SD, Knorr M, Parrent JL, Simpson RT. 2004. Chronic nitrogen enrichment affects the structure and function of the soil microbial community in temperate hardwood and pine forests. *Forest Ecology and Management* **196**: 159–171.

Fujyoshi L., Sugimoto A, Tsukuura A, Kitayama A, Lopez CML, Mijidsuren B, Saraadanbaza A, Tsujimura M. 2016. Spatial variations in larch needle and soil $\delta^{15}N$ at a forest-rassland boundary in northern Mongolia. *Isotopes in Environmental and Health Studies*, DOI: 10.1080/10256016.2016.1206093.

Fukasawa Y, Osono T, Takeda H. 2009. Microfungus communities of Japanese beech logs at different stages of decay in a cool temperate deciduous forest. *Can. J. For. Res.* **39**: 1606-1614.

Fukushima, T.; Takasuna, H.; Matsui, T.; Nishio, T.; Kyan, T.; Tsunetomi, Y. 1995. New phytosociological classification of beech forests in Japan. *Jpn. J. Ecol.* **45**: 79-98 (in Japanese with English synopsis).

Fukushima K, Tateno R, Tokuchi N. 2011. Soil nitrogen dynamics during stand development after clear-cutting of Japanese cedar (*Cryptomeria japonica*) plantations. *J For Res* **16:** 394-404.

Gan H, Jia, Y, Jia J, Wang X, Li H, Shi W, Peng C, Polle A, Luo Z. 2015. Phosphorus and nitrogen physiology of two contrasting poplar genotypes when exposed to phosphorus and/or nitrogen starvation. *Tree physiol*. **36** (1): 22-38.

Gebauer G, Schulze ED. 1991. Carbon and nitrogen isotope ratios in different compartments of a healthy and declining *Picea abies* forest in the Fichtergebirge, NE Bavaria. *Oecologia* **87**: 198-207.

Gebauer G & Dietrich P. 1993. Nitrogen Isotope Ratios in Different Compartments of a Mixed Stand of Spruce, Larch and Beech Trees and of Understorey Vegetation Including Fungi. *Isotopes in Environmental and Health Studies* **29**: 35-44.

Gebauer G, Giesemann A, Schulze ED, Jager HJ. 1994. Isotope ratios and concentrations of sulfur and nitrogen in needles and soils of *Picea abies* stands as influenced by atmospheric deposition of sulfur and nitrogen compounds. *Plant and Soil* **164**: 267-281.

Gebauer T, Horna V, Leuschner C. 2008. Variability in radial sap flux density patterns and sapwood area among seven co-occurring temperate broad-leaved tree species. *Tree Physiol.* **28:** 1821–1830.

Gessler A, Schenider S, von Sengbusch D, Weber P, Hanemann U, Huber C, Rothe A, Kreutzer K, Remmemnerg H. 1998a. Field and laboratory experiments on net uptake of nitrate and ammonium by the roots of spruce (*Picea abies*) and beech (*Fagus sylvatica*) trees. *New Phytologist* **138**: 275-285.

Gessler A, Schlutze M, Schrempp S, Rennenberg H. 1998b. Interaction of phloem-translocated amino compounds with nitrate net uptake by roots of beech (*Fagus sylvatica*) seedlings. *J. Exp. Bot.* **45**: 1529-1537.

González-Zurdo P, Escuerdo A, Mediavilla S. 2015. N resorption efficiency and proficiency in response to wintercold in three evergreen species. *Plant Soil* **394**: 87–98.

Gough CM. 2012. Terrestrial primary production: fuel for life. *Nat. Educ. Knowl.* **3**: 28

Guak S, Neilsen D, Millard P, Wendler R, Neilsen GH 2003. Determining the role of N remobilization for growth of apple (*Malus domestica* Borkh.) trees by measuring xylem-sap N flux. *Journal of Experimental Botany* Vol. **54**, 390: 2121-2131.

Güsewell, S. 2004. N:P ratios in terrestrial plants: Variation and functional significance. *N. Phytol.* **164**: 243–266.

Güsewell S & Gessner MO. 2009. N : P ratios influence litter decomposition and colonization by fungi and bacteria in microcosms. *Functional Ecology* **23**: 211–219.

Grassi G, Millard P, Wendler R, Minotta G, Tagliavini M. 2002. Measurement of xylem sap amino acid concentrations in conjunction with whole tree transpiration estimates spring

N remobilization by cherry (*Prunus avium* L.) trees. *Plant, Cell and Environment* **25**: 1689-1699.

Han Q, Kawasaki T, Nakano T, Chiba Y. 2008a. Leaf-age effects on seasonal variability in photosynthetic parameters and its relationships with leaf mass per area and leaf nitrogen concentration within a *Pinus densiflora* crown. *Tree Physiol*. **28**: 551-558.

Han Q, Kabeya D, Iio A, Kakubari Y. 2008b. Masting in *Fagus crenata* and its influence on the nitrogen content and dry mass of winter buds. *Tree Physiol.* **28**: 1269-1276.

Han Q, Kabeya D, Iio A, Inagaki Y, Kakubari Y. 2014. Nitrogen storage dynamics are affected by masting events in *Fagus crenata. Oecologia* **174**: 679–687

Harrison MT, Edwards EJ, Farqhuhar GD, Nicotra AB, Evans JR. 2009. Nitrogen in cell walls of sclerophyllous leaves accounts for little of the variation in photosynthetic nitrogen use efficiency. *Plant Cell Environ*. **32**: 259-270.

Hartmann M, Howes CG, VanInsberghe D, Yu H, Bachar D, Christen R, Henrik Nilsson R, Hallam SJ, Mohn WW. 2012. Significant and persistent impact of timber harvesting on soil microbial communities in Northern coniferous forests. *ISME J.* **6**: 2199–2218. http://dx.doi.org/10.1038/ismej.2012.84.7

Hayashi M, Lopez CML, Nobori Y, Byambasuren M, Boy J. 2018. Nitrogen isotope pattern in Mongolian Larch stands at the southern Eurasian boreal forest boundary. *Isotopes in Environmental and Health studies* **54** (6): 608-621.

Helmisaari HS. 1992. Nutrient retranslocation within the foliage of *Pinus sylvestris*. *Tree Physiol.* **10**: 45-58.

Heuer B. 1999. Osmoregulatory Role of Proline in Plants Exposed to Environmental Stresses. In: *Handbook of Plant and Crop Stress second Edition, Revised and Expanded.* New York, USA: Marcel Dekker, Inc., 675-695

Hobbie E & Ouimette AP. 2009. Controls of nitrogen isotope patterns in soil profiles. *Biogeochemistry* **95**: 355-371.

Hobbie EA, Agerer R. 2010. Nitrogen isotopes in ectomycorrhizal sporocarps correspond to belowground exploration types. *Plant Soil* **327**: 71-83.

Hobbie EA & Högberg P. 2012. Nitrogen isotopes link mycorrhizal fungi and plants to nitrogen dynamics. *New Phytologist* **196**: 367-382.

Holmberg M, Aherne J, Austnes K, Beloica J, De Marco A, Dirnböck T, Fornasier MF, Goergen K, Futter M, Lindroos A, Krám P, Neirynck J, Nieminen TM, Pecka T, Posch M, Pröll G, Rowe EC, Scheuschner T, Schlutow A, Valiniar S, Forsius M. 2018. Modelling study of C, N and pH response to air pollution and climate change using European LTER site observations. *Science of Total Environment* **640-641**: 387-399.

Hosoda K, Iehara T. 2010. Aboveground biomass equations for individual trees of *Cryptomeria japonica*, *Chamaecyparis obtusa* and *Larix kaempferi* in Japan. *J. For. Res*. **15**: 299-306.

Högberg P, Johannisson C, Yarwood S, Callesen I, Näsholm T, Myrold DD, Högberg MN. 2011. Recovery of ectomycorrhizal after 'nitrogen saturation' of a conifer forest. *New Phytologist* **189**: 515-525.

Högberg P, Johannisson C, Högberg MN. 2014. Is the high ^{15}N natural abundance of trees in N-loaded forests caused by an internal ecosystem N isotope redistribution or a chance in the ecosystem N isotope mass balance? *Biochemistry* **117**: 351-358.

Ishida A, Diloksumpun S, Ladpala P, Staporn D, Panuthai S, Gamo M, Yazaki K, Ishizuka M, Puangchit L. 2006. Contrasting seasonal leaf habits of canopy trees between tropical dry-deciduous and evergreen forests in Thailand. *Tree Physiology* **26**: 643-656.

Ishida TA, Nara K, Hogetsu T. 2007. Host effects on ectomycorrhizal fungal communities: insight from eight host species in mixed conifer–broadleaf forests *New Phytologist* **174**: 430-440.

Johnson EA. 2013. Biotechnology of non-*Saccharomyces* yeasts—the ascomycetes. *Applied Microbiology and Biotechnology* **97** (2): 503-517.

Jones DL, Shannon D, Murphy DV, Farrar J. 2004. Role of dissolved organic nitrogen (DON) in soil N cycling in grassland soils. *Soil Biology & Biochemistry* **36**: 749–756.

Jones DL & Willett VB. 2006. Experimental evaluation of methods to quantify dissolved organic nitrogen (DON) and dissolved organic carbon (DOC) in soil. *Soil Biology & Biochemistry* **38**: 991-999.

Jumpponen A, Jones KL, Mattox JD, Yaege C, Mattox JD, Yaege C. 2010.
Massively parallel 454-sequencing of fungal communities in Quercus spp. ectomycorrhizas indicates seasonal dynamics in urban and rural sites. *Molecular Ecology* **19**: 41–53.

Kai H & Iba K. 2014. Temperature Stress in Plants. eLS, doi:10.1002/9780470015902.a0001320.pub2

Kang H, Xin Z, Berg B, Burgess PJ, Liu Q, Liu Z, Li Z, Liu C. 2010. Global pattern of leaf litter nitrogen and phosphorus in woody plants. *Ann. For. Sci.* **67**: 811. DOI:10.1051/forest/2010047.

Kayama M, Makoto K, Nomura M, Satoh F, Koike T. 2009. Nutrient dynamics and carbon partitioning in larch seedlings (*Larix kaempferi*) regenerated on a serpentine soil in northern Japan. *Landscape Ecol. Eng.* **5**: 125-135.

Killingbeck KT. 1996. Nutrients in Senesced Leaves: Keys to the Search for Potential Resorption and Resorption Proficiency. *Ecology* **77** (6): 1716-1727.

Killingbeck KT. 2004. Nutrient resorption. *Plant cell death processes* (pp. 215-226).

Kim YK, Lim CS, Kang SM, Cho JL. 2009. Root storage of nitrogen applied in autumn and its remobilization to new growth in spring of persimmon trees (*Diospyros kaki* cv. Fuyu). *Scientia Horticulturae* **119**: 193-196.

Koba K, Tokuchi N, Wada E, Nakajima T, Iwatsubo G. 1997. Intermittent denitrification: The application of a ^{15}N natural abundance method to a forested ecosystem. *Gerchimica et Cosmochimica Acta* **61** (23): 5043-5050.

Koba K, Tokuchi N, Yoshioka T, Hobbie EA, Iwatsubo G. 1998. Natural Abundance of Nitrogen-15 in a Forest Soil. *Soil Science Society of America Journal* **62** (3): 778-781.

Koba K, Hirobe M, Koyama L, Kohzu A, Tokuchi N, Nadelhoffer KJ, Wada E, Takeda H. 2003. Natural ^{15}N Abundance of Plants and Soil N in a Temperate Coniferous Forest. *Ecosystems* **6**, 457-469.

Kobayashi H, Tashiro N. 2003. Temporal variation in leaf nitrogen content in a *Cryptomeria japonica* canopy. *The Japanese Society of Forest Environment* **45** Vol. 2:99-102.

Koide RT, Xu B, Sharda J, Lekberg Y, Ostiguy N. 2005. Evidence of species interactions within an ectomycorrhizal fungal community. *New Phytologist* **165**: 305–316.

Kolb KJ, Evans RD. 2002. Implications of leaf nitrogen recycling on the nitrogen isotope composition of deciduous plant tissues. *New Phytologist* **156**, Issue 1: 57-64.

Kõljalg U, Nilsson RH, Abarenkov K, Tedersoo L, Taylor AFS, Bahram M, Bates ST, Bruns TD, Bengtsson-Palme J, Callaghan TM, Douglas B, Drenkhan T, Eberhardt U, Dueñas M, Grebenc T, Griffith GW, Hartmann M, Kirk PM, Kohout P, Larsson E, Lindahl BD, Lücking R, Martín MP, Matheny PB, Nguyen NH, Niskanen T, Oja J, Peay KG, Peintner U, Peterson M, Põldmaa K, Saag L, Saar I, Schüßler A, Scott JA, Senés C, Smith ME, Suija A, Taylor DL, Telleria MT, Weiss M, Larsson K-H. 2013. Towards a unified paradigm for sequence-based identification of fungi. *Molecular Biology* **22** (21): 5271-5277.

Konôpka B, Noguchi K, Sakata T, Takahashi M, Konopkôvá Z. 2006. Fine root dynamics in a Japanese cedar (Cryptomeria Japonica) plantation throughout the growing season. *Forest Ecology and Management* **225**: 278-286.

Koerselman W & Meuleman AFM. 1991. The Vegetation N:P Ratio: a New Tool to Detect the Nature of Nutrient Limitation. *Journal of Applied Ecology* **33:** 1441-1450.

Kyaschenko J, Clemmensen KE, Karltun E, Lindahl BD. 2017. Below-ground organic matter accumulation along a boreal forest fertility gradient relates to guild interaction within fungal communities. *Ecology Letters* **20**: 1546–1555.

Lang C, Finkeldey R, Polle A. 2013. Spatial patterns of ectomycorrhizal assemblages in a monospecific forest in relation to host tree genotype. *Front. Plant. Sci*. **4**: Article 103, 1-9. https://doi.org/10.3389/fpls.2013.00103

Lea PJ, Sodek L, Parry MAJ, Shewry PR, Halford NG. 2007. Asparagine in plants*. Ann. Appl. Biol.* **150**: 1-26.

Leberecht M, Dannenmann M, Tejedor J, Simon J, Rennenberg H, Polle A. 2016. Segregation of nitrogen use between ammonium and nitrate of ectomycorrhizas and beech trees. *Plant Cell Environ.* **39**: 2691-2700.

Li H, Crabbe MJC, Xu F, Wang W, Niu R, Gao X, Zhang P, Chen H. 2017. Seasonal Variations in Carbon, Nitrogen and Phosphorus Concentrations and C:N:P Stoichiometry in the Leaves

of Differently Aged *Larix principis-rupprechtii* Mayr. Plantations. *Forests* **8**: 373, doi:10.3390/f8100373

Liang N, Nagayama M, Nakata M, Maruyama K. 1995. Growth, photosynthesis and nitrogen content in Japanese beech (*Fagus crenata* Bl.) seedlings grown under five irradiances. *Photosynthetica* **31** (2): 257-268.

Lim H, Lee K-H, Lee K-H, Park IH. 2013. Biomass expansion factors and allometric equations in an age sequence for Japanese cedar (*Cryptomeria japonica*) in southern Korea. *J For Res* **18**: 316-322.

Lin W-R, Wang P-H, Chen W-C, Lai C-M, Winder, RS. 2016. Responses of Soil Fungal Populations and Communities to the Thinning of *Cryptomeria Japonica* Forests. Microbes Environ. Doi:10.1264/jsme2.ME15127

Lopez CML, Mizota C, Yamanaka T, Nobori Y. 2010. Temporal changes in tree-ring nitrogen of *Pinus thunbergii* trees exposed to Black-tailed Gull (*Larus crassirostris*) breeding colonies. *Appl. Geochem.* **25**: 1699-1702.

Lopez CML, Mizota C, Nobori Y, Sasaki T, Yamanaka T. 2014. Temporal changes in nitrogen acquisition of Japanese black pine (*Pinus thunbergii*) associated with black locust (*Robinia pseudo acacia). Journal of Forestry Research* **25** (3): 585-589.

Lopez CML, Nakano S, Ferrio JP, Hayashi M, Nakatsuka T, Sano M, Yamanaka T, Nobori Y. 2018. Evaluation of the effect of the 2011 Tsunami on coastal forests by means of multiple isotopic analyses of tree rings. *Isotopes in Environmental and Health studi*es **54** (5) 494-507.

Luo J; Zhou J; Li H; Shi W; Polle A; Lu M; Sun X; Luo Z. 2015. Global poplar root and leaf transcriptomes reveal links between growth and stress responses under nitrogen starvation and excess. *Tree Physiol.* tpv091

Mae T. 2004. Leaf senescence and nitrogen metabolism. In: *Noodén LD (ed) Plant cell death processes.* New York, USA: Academic Press, 157-168.

Malagoli M, Dal Canal A, Quaggiotti S, Pegoraro P, Bottacin A. 2000. Differences in nitrate and ammonium uptake between Scots pine and European larch. *Plant and Soil* **221**: 1-3.

Malaguti D, Millard P, Wendler R, Hepburn A, Tagliavini M. 2001. Translocation of amino acids in the xylem of apple (*Malus domestica* Borkh.) trees in spring as a consequence of both N remobilization and root uptake. *Journal of Experimental Botany* **52** (361): 1665-1671.

Malcolm GM, Bush DS, Rice SK. 2008. Soil nitrogen conditions approach preinvasion levels following restoration of nitrogen-fixing black locust (*Robinia pseudoacacia*) stands in a pine-oak ecosystem. *Restor Ecol*, **16:** 70−78.

Mailk V, Timmer VR. 1997. Biomass partitioning and nitrogen retranslocation in black spruce seedlings on competitive mixed wood sites: a bioassay study. *Can. J of For. Res.* **28**: 206-214.

Marschner, H.1995. Mineral Nutrition of Higher Plants. Academic Press, London, pp. 889.

Marx F. 2004. Small, basic antifungal proteins secreted from filamentous ascomycetes: a comparative study regarding expression, structure, function and potential application. *Applied Microbiology and Biotechnology* **65** (2): 133-142.

Matsui, H. 1995. Effects of acidity of the water culture solution on uptake of nutrients by the root of Japanese cedat (*Cryptomeria japonica*) and Japanese cypress (*Chamaecyparis obtusa*). *Water, Air and Soil Pollution* **85**: 1131-1136.

McKinley DC, Ryan MG, Birdsey RA, Giardina CP, Harmon ME, et al. 2011. A synthesis of current knowledge on forests and carbon storage in the United States.*Ecol. Appl.* **21**: 1902–24.

Mead DJ, Preston CM. 1994. Distribution and retranslocation on ^{15}N lodge pole pine over eight growing seasons. *Tree Physiol.* **14**: 389-402.

Meerts P. 2002. Mineral nutrient concentrations in sapwood and heartwood: a literature review. *Ann. For. Sci.* **59**: 713–722.

Mengel K. 1991. Ernährung und Stoffwechsel der Pflanze. *Gustav Fischer Verlag*, Jena, p 324-334. In German.

Michalzik B & Matzner E. 1999. Dynamics of dissolved organic nitrogen and carbon in a Central European Norway spruce ecosystem. *Soil Science* **50** (4): 579-590.

Millard P, Proe M. 1992. Storage and internal cycling of nitrogen in relation to seasonal growth of Sitka spruce. *Tree Physiol.* **10**: 33-43.

Millard P, Proe M. 1993. Nitrogen uptake, partitioning and internal cycling in *Picea sitchensis* (Bong.) Carr. As influenced by nitrogen supply. New *Phytologist* **125**: 113-119.

Millard P, Hester A, Wendler R, Baillie G. 2001. Interspecific defoliation responses of trees depend on sites of winter nitrogen storage. *Functional Ecology* **15**: 535-543.

Millard P, Wendler R, Grassi G, Grelet GA, Tagliavini M. 2006. Translocation of nitrogen in the xylem of field-grown cherry and poplar trees during remobilization. *Tree Physiology* **26**: 527-536.

Millard P, Grelet G-A. 2010. Nitrogen storage and remobilization by trees: ecophysiological relevance in a changing world. *Tree Physiology* **30:** 1083-1098.

Miranda KM, Espey MG, Wink DA. 2001. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. *Nitric Oxide – Biology and Chemistry* **5**: 62-71.

Miyashita Y, Dolferus R, Ismond KP, Goog, AG. 2007. Alanine aminotransferase catalyses the breakdown of alanine after hypoxia in *Arabidopsis thaliana*. *The plant journal* **49** (6): 1108-1121.

Miyamoto Y, Nakano T, Hattori M, Nara K. 2014. The mid-domain effect in ectomycorrhizal fungi: range overlap along an elevation gradient on Mount Fuji, Japan. *The ISME Journal* **8**: 1739-1746.

Miyazaki Y, Maruyama Y, Chiba Y, Kobayashi MJ, Joseph B, Shimizu KK, Mochida K, Hiura T, Kon H, Satake A. 2014. Nitrogen as a key regulator of flowering in *Fagus crenata*: understanding the physiological mechanism of masting by gene expression analysis. *Ecology Letters* **17**: 1299-1309.

Mizota C, Lopez CML, Yamanaka T, Nobori Y. 2011. Differential response of two *Pinus* spp. to avian nitrogen input as revealed by nitrogen isotope analysis for tree rings. *Isot. Environ. Health Stud.* **47**: 62-70.

Moon HS, Haruki M. 1999. Nutrient Distribution and Content in Plant Tissue, Forest Floor, and Litterfall in Four Pioneer Stands on Volcano Mt. Showa-Shinzan. *Jpn. J. For. Environment* **41** (1): 7-11.

Mori T, Ohta S, Ishizuka S, Konda R, Wicaksono A, Heriyanto J, Hamotani Y, Gobara Y, Kawabata C, Kuwashima K, Nakayama Y, Hardjono A. 2013. Soil greenhouse gas fluxes and C stocks as affected by phosphorus addition in a newly established *Acacia mangium* plantation in Indonesia. *For. Ecol. Manage.* **310**: 643–651.

Moser MA, Petersen CA, D'Allura JA, Southworth D. 2005. Comparison of ectomycorrhizas of *Quercus garryana* (Fagaceae) on serpentine and nonserpentine soils in southwestern Oregon. *American Journal of Botany* **92**: 224–230.

Muller B, Touraine B, Renenberg H. 1996. Interaction between atmospheric and pedospheric N nutrition in spruce (Picea abies (L.) Karst.) seedlings. *Plant cell environ.* **19**: 345-355.

Mulvaney RL. 1996. Nitrogen – Inorganic forms: Methods of Soil Analysis. Part 3. Chemical methods, SSSA, Madison, WI, USA. Pp. 125-1184.

Nakaji T, Fukami M, Dokiya Y, Izuta T. 2001. Effects of high nitrogen load on growth, photosynthesis and nutrient status of *Cryptomeria japonica* and *Pinus densiflora* seedlings. *Trees* **15:** 453-461.

Nambiar EKS & Fife DN. 1991. Nutrient retranslocation in temperate conifers. *Tree physiol.* **6**: 267-281.

Nantel P, Neumann P. 1992. Ecology of ectomycorrhizal-basidiomycete communities on a local vegetation gradient. *Ecology* **73**: 99–117.

Nara K, Nakaya H, Wu B, Zhou Z, Hogetsu T. 2003. Underground primary succession of ectomycorrhizal fungi in a volcanic desert on Mount Fuji. *New Phytologist* **159**: 743–756.

Nara K. 2006. Pioneer dwarf willow may facilitate tree succession by providing late colonizers with compatible ectomycorrhizal fungi in a primary successional volcanic desert. *New Phytologist* **171**: 187-198.

Näsholm T & Ericsson A. 1990. Seasonal changes in amino acids, protein and total nitrogen in needles of fertilized Scots pine trees. *Tree Physiol.* **6:** 267–281.

Näsholm T. 1994. Removal of nitrogen during needle senescence in Scots pine (*Pinus sylvestris L*.). *Oecologia* **99**: 290-296.

Neff JC & Hooper DU. 2002. Vegetation and climate controls on potential CO_2, DOC and DON production in northern latitude soils. *Global Change Biology* **8**: 872-884.

Nguyen NH, Song Z, Bates ST, Branco S, Tedersoo L, Menke J, Schilling JS, Kennedy PG. 2016. FUNGuild: An open annotation tool for parsing fungal community datasets by ecological guild. *Fungal Ecology* **20**: 241-248.

Nordin A, Uggla C, Näsholm T. 2001. Nitrogen forms in bark, wood and foliage of nitrogen-fertilized Pinus sylvestris. *Tree Physiol.* **21**: 59-64.

O'Brien HE, Parrent JL, Jackson JA, Moncalvo J-M,Vilgalys R. 2005. Fungal Community Analysis by Large-Scale Sequencing of Environmental Samples. *Applied and Environmental Microbiology* **71** (9): 5544-5550.

Ochimaru T, Fukuda K. 2007. Changes in fungal communities in evergreen broad-leaved forests across a gradient of urban to rural areas in Japan. *Can. J. For. Res*. **37**: 247-258.

O'Dell TE, Ammirati JF, Schreiner EG. 1999. Species richness and abundance of ectomycorrhizal basidiomycete sporocarps on a moisture. *New Phytologist* (2007) **174**: 430–440

Ohashi M, Gyokusen K, Saito A. 1999. Measurement of carbon dioxide evolution from a Japanese cedar (*Cryptomeria japonica* D. Don) forest floor using and open-flow chamber method. *Forest Ecology and Management* **12:** 105-114.

Oikawa A, Ostuka T, Nakabayashi R, Jikumaru Y, Isuzugawa K, Murayama H, Saito K, Shiratake K. 2015. Metabolic Profiling of Developing Pear Fruits Reveals Dynamic Variation in Primary and Secondary Metabolites, Including Plant Hormones. *PLoS One* **10** (7): e0131408. https://doi.org/10.1371/journal.pone.0131408.

Oksanen, J., Blanchet, F., Kindt, R., Legendre, P., O'Hara, R., Simpson, G., Solymos, P.,Stevens, M., Wagner, H., 2018. Vegan: Community Ecology Package. R PackageVersion 1.17-8. Available from: http://CRAN.R-project.org/package-vegan.

Olander LP, Vitousek PM. 2000. Regulation of soil phosphatase and chitinase activity by N and P availability. *Biochemistry* **49**: 175-190.

Oliveira HC, Inocêncio da Silva LM, Dias de Freitas L, Debiasi TV, Marchiori N M, Aidar MPM, Bianchini E, Pimenta JA, Stolf-Moreira R. 2017. Nitrogen use strategies of seedlings from neotropical tree species of distinct successional groups. *Plant Physiology and Biochemistry* **114**: 119-127.

Olsen SR, Cole CV, Watanabe FS, Dean LA. 1954. Estimation of available phosphorus in soils by extraction with sodium bicarbonate. Washington, D.C.: U.S. Government Printing Office.

Ono K, Yasuda Y, Matsuo T, Hoshino D, Chiba Y, Mori S. 2013. Estimating forest biomass using allometric model in a cool-temperate Fagus crenata forest in the Appi highlands, Iwate, Japan. *For. Res. Inst. Res. Rep.* **12**: 125–141.

Osada N., Yasumura Y., Ishida A. 2014. Leaf nitrogen distribution in relation to crown architecture in the tall canopy species, *Fagus crenata*. *Oecologia* **175**: 1093-1106.

Özbucak TB, Kutbay HG, Kliic D, Korkmaz H, Bilgin A, Yalçin E, Apaydin Z. 2008. Foliar resorption of nutrients in selected sympatric tree species in gallery forest (Black Sea Region). *Polish Journal of Ecology* **56**: 227-237.

Palacio S, Camarero JJ, Maestro M, Alla AQ, Lahoz E, Montserrat-Martí G. 2018. Are storage and tree growth related? Seasonal nutrient and carbohydrate dynamics in evergreen and deciduous Mediterranean oaks. *Trees* **32** (3): 777-790.

Pan Y, Birdsey RA, Fang J, Houghton R, Kauppi PE, et al. 2011. A large and persistent carbon sink in the world's forests. *Science* **333:** 988–93

Pan Y, Birdsey RA, Phillips OL, Jackson RB. 2013. The Structure, Distribution, and Biomass of the World's Forests. *Annu. Rev. Ecol. Evol. Syst..* **44**: 593–622.

Pastor J, Aber JD, McClaugherty CA, MelilloJM. 1984. Aboveground Production and N and P Cycling Along a Nitrogen Mineralization Gradient on Blackhawk Island, Wisconsin. *Ecology* **65** (1): 256-268.

Pâques LE. 2001. Genetic Control of Heartwood Content in Larch. *Silvae Genetica* **50** (2): 69-75.

Peri PL, Ladd B, Pepper DA, Bonser SP, Laffan SW, Amelung W. 2012. Carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotope composition in plant and soil in Southern Patagonia's native forests. *Global Change Biology* **18**: 311-321.

Pietilä M, Lähdesmäki P, Ferm A, Hytönen J, Pätilä A. 1991. High nitrogen deposition causes changes in amino acid concentrations and protein spectra in needles of scots pine (*pinus sylvestris*). *Environ. Pollut.* **72**: 103-115.

Pinheiro J, Bates D, DebRoy S, Sarkar D, R Development Core Team. 2013. NLME: Linear and nonlinear mixed effects models. In R package version 3.1-109.

Plassard C, Dell B. 2010. Phosphorus nutrition of mycorrhizal trees. *Tree Physiology* **30**: 1129–1139.

Prober SM, Leff JW, Bates ST, Borer ET, Firn J, Harpole WS, Lind EM, Seabloom EW, Adler PB, Bakker JD, Cleland EE, DeCrappeo NM, DeLorenze E, Hagenah N, Hautier Y, Hofmockel KS, Kirkman KP, Knops JM, La Pierre KJ, MacDougall AS, McCulley RL, Mitchell CE, Risch AC, Schuetz M, Stevens CJ, Williams RJ, Fierer N. 2014. Plant diversity predicts beta but not alpha diversity of soil microbes across grasslands worldwide. *Ecol Lett.* **18**(1):85-95.

Proe M, Millard P. 1994. Relationships between nutrient supply, nitrogen partitioning and growth in young Sitka spruce (*Picea sitchensis*). *Tree Physiol.* **14**: 75-88.

Proe MF, Midwood AJ, Craig J. 2000. Use of stable isotopes to quantify nitrogen, potassium and magnesium dynamics in young Scots pine (*Pinus sylvestris*). *New Phytologist* **146**: 461-469.

R Development Core Team. 2019. R: A language and environment for statistical computing.Vienna: R Foundation for Statistical Computing.

Reich PB & Oleksyn J. 2004. Global patterns of plant leaf N and P in relation to temperature and latitude. *PNAS* **101** (30): 11001-11006.

Rennenberg H & Herschbach C. 2013. Phosphorus nutrition of woody plants: many questions – few answers. *Plant biology* **15**: 785-788.

Rennenberg H & Dannenmann M. 2015. Nitrogen nutrition of trees and temperate forests – the significance of nitrogen availability in pedosphere and atmosphere. *Forests* **6**: 2820–2835.

Rhodes CJ. 2017. The whispering world of plants: 'The wood wide web'. *Science Progress* **100** (3): 331 – 337

Rice SK, Westerman B, Federici R. 2004. Impacts of the exotic, nitrogen-fixing black locust (*Robinia pseudoacacia*) on nitrogen cycling in a pine-oak ecosystem. *Plant Ecol*, **174**: 97−107.

Richardson SJ, Peltzer DA, Allen RB, McGlone MS. 2005. Resorption proficiency along a chronosequence: Responses among communities and within species. *Ecology* **86** (1): 20-25.

Roberts P & Jones DL. 2012. Microbial and plant uptake of free amino sugars in grassland soils. *Soil Biol. Biochem.***49:** 139–149.

Robinson D. 2001. $\delta^{15}N$ as an integrator of the nitrogen cycle. *TRENDS in Ecology & Evolution* Vol **16** No.3: 153-162.

Rosecrance RC, Weinbaum SA, Brown PH. 1998. Alternate Bearing Affects Nitrogen, Phosphorus, Potassium and Starch StoragePools in Mature Pistachio Trees. *Annals of Botany* **82**: 463-470.

Rousk J, Brookes PC, Bååth E. 2009. Contrasting Soil pH Effects on Fungal and Bacterial Growth Suggest Functional Redundancy in Carbon Mineralization. *Applied and Environmental Microbiology* **75** (6): 1589-1596.

Rousk J, Bååth E, Brookes PC, Lauber CL, Lozupone C, Caporaso JG, Knight R, Fierer N. 2010. Soil bacterial and fungal communities across a pH gradient in an arable soil. *The ISME Journal* **4**: 1340-1351.

Rousk J, Brookes PC, Bååth E. 2011. Fungal and bacterial growth responses to N fertilization and pH in the 150-year 'Park Grass' UK grassland experiment. *FEMS Microbiology Ecology* **76** (1): 89–99.

Ruddell S, Sampson R, Smith M, Giffen R, Cathcart J, Hagan J, Sosland D, Godbee J, Heissenbuttel J, Lovett S, Helms J, Price W, Simpson R. 2007. The Role for Sustainably Managed Forests in Climate Change Mitigation. *Journal of Forestry* **105** (6): 314–319.

Sakai Y, Takahashi M, Tanaka N. 2007. Root biomass and distribution of a *Picea–Abies* stand and a *Larix–Betula* stand in pumiceous Entisols in Japan. *J. For. Res.* **12**: 120-125.

Saiya-Cork KR, Sinsabaugh RL, Zak DR. 2002. The effects of long term nitrogen deposition on extracellular enzyme activity in an *Acer saccharum* forest soil. *Soil Biology and Biochemistry* **34** (9): 1309-1315.

Santalahti M, Sun H, Jumpponen A, Pennanen T, Heinonsalo J. 2016. Vertical and seasonal dynamics of fungal communities in boreal Scots pine forest soil. *FEMS Microbiol. Ecol.* **92**, fiw170.

Santiago LS; Wright SJ; Harms KE; Yavitt JB; Korine C; Garcia MN; Turner BL. 2012. Tropical tree seedling growth responses to nitrogen, phosphorus and potassium addition*. J. Ecol.* **100**: 309–316.

Sardans, J., Peñuelas, J., 2013. Plant-soil interactions in Mediterranean forest and shrublands: impacts of climatic change. *Plant Soil* **365**: 1–33.

Sasse J. 1998. *The Forests of Japan*. Tokyo, Japan: Japan Forest Technical Association 1998.

Schmitz A, Sanders TGM, Bolte A, Bussotti F, Dirnböck T, Johnson J, Peñuelas J, Pollastrini M, Prescher A-K, Sardans J, Verstraeten A, de Vries W. 2019. Responses of forest ecosystems in Europe to decreasing nitrogen deposition. *Environmental Pollution* **244**: 980–994.

Schneider S, Geßler A, Weber P, von Sengbusch D, Hanemann U, Rennenberg H. 1996. Soluble N compounds in trees exposed to high loads of N: a comparison of spruce (*Picea abies)* and beech (*Fagus sylvatica*) grown under field conditions. *New Phytologist* **134**: 103-114.

Seidel F, Lopez CML, Guggenberger G, Nobori Y. 2017. Impact of a low severity fire on soil organic carbon and nitrogen characteristics in Japanese cedar soil, Yamagata Prefecture, Japan. *Open Journal of Forestry* **7**: 270-284.

Seidel F, Lopez CML, Oikawa A, Yamanaka T. 2019a. Seasonal nitrogen partitioning in Japanese cedar (*Cryptomeria japonica*, D. Don) tissues. (In press).

Seidel F, Lopez CML, Celi L, Bonifacio E, Oikawa A, Yamanaka T. 2019b. Tree tissues N isotope fractionation during N intra mobilization in *Fagus crenata*. *Forests* **10**, 330 doi:10.3390/f10040330

Seidel F, Lopez CML, Celi L, Bonifacio E, Oikawa A, Yamanaka T. 2019c. Nitrogen partitioning in Japanese larch (*Larix kaempferi*) and Black locust (*Robinia pseudoacacia*) at four phenological stages revealed by free amino acids. *In preparation*.

Shi J, Ohte N, Tokuchi N, Imamura N, Nagayama M, Oda T, Suzuki M. 2014. Nitrate isotopic composition reveals nitrogen deposition and transformation dynamics along the canopy-soil continuum of a suburban forest in Japan. *Rapid Communications in Mass Spectrometry* **28**: 2539-2549.

Shibata H, Branquinho C, McDowell WH, Mitchell MJ, Monteith DT, Tang J, Arvola L, Cruz C, Cusack DF, Halada L, Kopáček J, Máguas C, Sajidu S, Schubert H, Tokuchi N, Zájora. 2015. Consequence of altered nitrogen cycles in the coupled human and ecological system under changing climate: The need for long-term and site-based research. *AMBIO* **44**: 178-193.

Shilenkova OL, Tiunov AV. 2013. Soil-litter nitrogen transfer and changes in $\delta^{13}C$ and $\delta^{15}N$ values in decomposing leaf litter during a laboratory incubation. *Pedobiologia* **56**: 147-152.

Shimano K & Masuzawa T. 1998. Effects of snow accumulation on survival of beech (*Fagus crenata*) seed. *Plant Ecology* **134**: 235-241.

Silla F, Escudero A. 2003. Uptake, demand and internal cycling of nitrogen in saplings of Mediterranean *Quercus* species. *Oecologia* **136**: 28-36.

Singh A. (2014). Nitrogen and phosphorus resorption efficiency in some, native tropical trees planted on a mine spoil in Singrauli Coalfields, India. *International Journal of Environment and Bioenergy* **9** (3): 161-170.

Singh A. (2015). Nitrogen and phosphorus resorption efficiency in some leguminous and non-leguminous tropical tree species planted on coal mine spoil in a tropical dry environment. *Ambit J Educ Res Rev* **1**: 1-7.

Six J. Frey SD, Thiet RK, Batten KM. 2006. Bacterial and Fungal Contributions to Carbon Sequestration in Agroecosystems. *Soil Biology & Biochemistry* **70** (2): 555-569.

Smith SE & Read DJ. 2008. Mycorhizal symbiosis, 3rd edition. Elsevier/Academic Press, Amsterdam, The Netherlands.

Son Y & Gower ST. 1992. Nitrogen and phosphorus distribution for five plantation species in south western Wisconsin. *Forest Ecology and Management* **53** (1-4): 175-193.

Son Y, Lee IK, Ryu SR. 2000. Nitrogen and Phosphorus Dynamics in Foliage and Twig of Pitch Pine and Japanese Larch Plantations in Relation to Fertilization. *Journal of Plant Nutrition* **23** (5): 697-710.

Szpak P. 2014. Complexities of nitrogen isotope biochemistry in plant-soil systems: implications for the study of ancient agricultural and animal management practices. *Frontiers in Plant Science* Vol. **5**, Article 288: 1-19.

Swan HSD. 1971. Relationships between nutrient supply, growth and nutrient concentrations in the foliage of white and red spruce. *Pulp Pap. Res. Inst. Can.*, Woodlands Pap. WR/**34**. 27 p.

Tagliavini M, Millard P, Quartieri M, Marangoni B. 1999. Timing of nitrogen uptake affects winter storage and remobilizationof nitrogen in nectarine (*Prunus persica* var. *nectarina*) tress. *Plant Soil* **211**: 149-153.

Takebayashi Y, Koba K, Sasaki Y, Fang Y, Yoh M. 2010. The natural abundance of ^{15}N in plant soil-available N indicates a shift of main plant N resources to NO_3^- from NH_4^+ along the N leaching gradient. *Rapid Commun. Mass Spectrom*. **24**: 1001-1008.

Taniguchi T, Kanzaki N, Tamai S, Yamanaka N, Futai K. 2007. Does ectomycorrhizal fungal community structure vary along a Japanese black pine (*Pinus thunbergii*) to black locust (*Robinia pseudoacacia*) gradient? *New Phytologist* **173**: 322-334.

Tateno R, Osada N, Terai M, Tokuchi N, Takeda H. 2005. Inorganic nitrogen source utilization by *Fagus crenata* on different soil types. *Trees* **19**: 447-481.

Tateishi M, Kumagai T, Suyama Y, Hiura T. 2010. Differences in transpiration characteristics of Japanese beech trees, *Fagus crenata*, in Japan. *Tree Physiol.* **30**: 748–760.

Templer PH, Arthur MA, Lovett GM, Weathers KC. 2007. Plant and soil natural abundance $\delta^{15}N$: indicators of relative rates of nitrogen cycling in temperate forest ecosystems. *Oecologica* **153:** 399-406.

Tian C, He X, Zhong Y, Chen J. 2003. Effect of inoculation with ecto- and arbuscular mycorrhizae and *Rhizobium* on the growth and nitrogen fixation by black locust, *Robinia pseudoacacia. New Forests* **25**: 125-131.

Titus JS, Kang SM. 1982. Nitrogen metabolism, translocation, and recycling in apple trees. *Hort. Rev.* **4**: 204-246.

Tomlinson G, Siegwolf RTW, Buchmann N, Schleppi P, Waldner P, Weber P. 2014. The mobility of nitrogen across tree-rings of Norway spruce (*Picea abies* L.) and the effect of extraction method on tree-ring $\delta^{15}N$ and $\delta^{13}C$ values. *Rapid communications in Mass Sprectrometry* **28**: 1258-1264.

Turner BL. 2008. Resource partitioning for soil phosphorus: a hypothesis. *Journal of Ecology* **96**: 698-702.

Ueda MU, Mizumachi E, Tokuchi N. 2011. Foliage nitrogen turnover: differences among nitrogen absorbed at different times by Quercus serrata saplings. *Ann. Bot*. **108**: 169-175.

Van Heerwaarden, LM, Toet S, Aerts R. 2003. Current measures of nutrient resorption efficiency lead to a substantial underestimation of real resorption efficiency: facts and solutions. *Oikos*, **101** (3): 664-669.

Verbruggen N, Hermans C. 2008. Proline accumulation in plants: a review. *Amino Acids* **35**: 753-759.

Vergutz L, Manzoni S, Porporato A, Novais RF, Jackson RB. 2012. Global resorption efficiencies and concentrations of carbon and nutrients in leaves of terrestrial plants. *Ecological Monographs* **82**(2): 205-220.

Vítková M, Müllerová J, Sádlo J, Pergl J, Pyšek P. 2017. Black locust (*Robinia pseudoacacia*) beloved and despised: A story of an invasive tree in Central Europe. *Forest Ecology and Management* **384**: 287-302.

Voříšková J, Brabcová V, Cajthaml T, Baldrian P. 2014. Seasonal dynamics of fungal communities in a temperate oak forest soil. *New Phytologist* **201**: 269–278.

Vuorenmaa J, Augustaitis A, Beudert B, Bochenek W, Clarke N, de Wit HA, Dirnböck T, Frey J, Hakola H, Kleemola S, Kobler J, Krám P, Lindroos A, Lundin L, Löfgren S, Marchetto A, Pecka T, Schulte-Bisping H, Skotak K, Srybny A, Szpikowski J, Ukonmaanaho L, Váňa M, Åkerbloom S, Forsius M. 2018. Longterm changes (1990-2015) in the atmospheric deposition and runoff water chemistry of sulphate, inorganic nitrogen and acidity for forested catchments in Europe in relation to changes in emissions and hydrometeorological conditions. *Science of Total Environment* **625**: 1129-1145

Walker JF, Miller OK, Horton JL. 2005. Hyperdiversity of ectomycorrhizal fungus assemblages on oak seedlings in mixed forests in the southern Appalachian Mountains. *Molecular Ecology* **14**: 829–838.

Wallander H, Nilsson LO, Hagerberg D, Baath E. 2001. Estimation of the biomass and seasonal growth of external mycelium of ectomycorrhizal fungi in the field. *New Phytologist* **151**: 753–760.

Wallenda T, Kottke I. 1998. Nitrogen deposition and ectomycorrhizas. *New Phytologist* **139**: 169–187.

Wang J., Zhang T, Li L, Li J, Feng Y, Lu Q. 2017. The Patterns and Drivers of Bacterial and Fungal β-Diversity in a Typical Dryland Ecosystem of Northwest China. *Front Microbiol.* **8**: 2126. doi:10.3389/fmicb.2017.02126

Waring BG, Becknell JM, Powers JS. 2015. Nitrogen, phosphorus, and cation use efficiency in stands of regenerating tropical dry forest. *Oecologia* **178**: 887-897.

Warren CR, Dreyer E, Adams MA. 2003. Photosynthesis-Rubisco relationships in foliage of Pinus sylvestris in response to nitrogen supply and the proposed role of Rubisco and amino acids as nitrogen stores. *Trees* **17**: 359-366.

Warren CR, Adams MA. 2004. Evergreen trees do not maximize instantaneous photosynthesis. *Trends Plant Sci.* **9**: 270-274.

Watanabe M, Yamaguchi M, Matsumura H, Kohno Y, Izuta T. 2012. Risk assessment of ozone impact on *Fagus crenata* in Japan: consideration of atmospheric nitrogen deposition. *Eur. J. Forest Res.* **131**: 475-484.

Weber P, Stoermer H, Geßler A, Schneider S, von Sengbusch D, Hanemann U, Renenberg H. 1998. Metabolic responses of Norway spruce (*Picea abies*) trees to long-term forest management practices and acute $(NH_4)_2SO_4$ fertilization: transport of soluble non-protein nitrogen compounds in xylem and phloem. *New Phytologist* **140**: 461-475.

Whittaker RH. 1960. Vegetation of the Siskiyou mountains, Oregon and California. *Ecol. Monogr.* **30**: 279–338. doi:10.2307/1948435

Windler RS, Shamoun SF. 2006. Forest pathogens: friend or foe to biodiversity? *Can. J. Plant Pathol*. **28**: S221-S227.

Wright IJ, Reich PB, Cornelissen JH, Falster DS, Garnier E, Hikosaka K, Lamont BB, Lee W, Oleksyn J, Osada N, Poorter H, Villar R, Warton DI, Westoby M. 2005. Assessing the generality of global leaf trait relationships. *New Phytol.* **166:** 485–496.

Wright SJ, Yavitt JB, Wurzburger N, Turner BL, Tanner EVJ, Sayer EJ, Santiago LS, Kaspari M, Hedin LO, Harms KE, Garcia MN, Corre MD. 2011. Potassium, phosphorus, or nitrogen limit root allocation, tree growth, or litter production in a lowland tropical forest. *Ecology* **92**: 1616–1625.

Wu B, Tian J, Bai C, Xiang M, Sun J, Liu X. 2013. The biogeography of fungal communities in wetland sediments along the Changjiang River and other sites in China. *ISME J.* **7**(7): 1299-309.

Wyka TP, Zytkowiak R, Oleksyn J. 2016. Seasonal dynamics of nitrogen level and gas exchange in different cohorts of Scots pine needles: a conflict between nitrogen mobilization and photosynthesis? *European Journal of Forest Research* **135**, Issue 3: 483-493.

Xu Y, Xiao H. 2016. Free amino acid concentrations and nitrogen isotope signatures in *Pinus massoniana* (Lamb.) needles of different ages for indication atmospheric nitrogen deposition. *Environmental Polllution* **221** (2017): 180-190.

Yasumura Y, Hikosaka K, Matsui K, Hirose T. 2002. Leaf-level nitrogen-use efficiency of canopy and understorey species in a beech forest. *Functional Ecology* **16**: 826-834.

Yasumura Y, Onoda Y, Hikosaka K, Hirose T. 2005. Nitrogen resorption from leaves under different growth irradiance in three deciduous woody species. *Plant Ecology*, **178** (1): 29-37.

Yavitt JB, Harms KE, Garcia MN, Mirabello MJ, Wright SJ. 2011. Soil fertility and fine root dynamics in response to 4 years of nutrient (N, P, K) fertilization in a lowland tropical moist forest, Panama Austral. *Ecology* **36**: 433–445.

Yuan, ZY, Li LH, Han XG, Huang JH, Jiang GM, Wan SQ, Zhang WH, Chen QS. 2005. Nitrogen resorption from senescing leaves in 28 plant species in a semi-arid region of northern China. *Journal of Arid Environments* **63**(1): 191-202.

Zhang S; Jiang H; Zhao H; Korpelainen H; Li C. 2014. Sexually different physiological responses of *Populus cathayana* to nitrogen and phosphorus deficiencies. *Tree Physiol.* **34**, 343–354.

Zhou Z & Hogetsu T. 2002. Subterranean community structure of ectomycorrhizal fungi under *Suillus grevillei* sporocarps in a *Larix kaempferi* forest. *New Phytologist* **154**: 529-539.

Zhou W-J, Sha L-Q, Schaefer DA, Zhang Y-P, Song Q-H, Tan Z-H, Deng Y, Deng X-B, Guan H-L. 2014. Direct effects of litter decomposition on soil dissolved organic carbon and nitrogen in a tropical rainforest. *Soil Biology & Biochemistry* **81**: 255-258.

Abstract in Japanese

研究背景と目的：窒素（N）とリン（P）は最も重要な植物栄養素であり、生態系の発展における推進力であり、植物生長を決定し制限する光合成機構において重要な役割を果たしている。N とP のサイクルは、微生物や植物が有機的に土壌中の栄養をミネラル化する酵素とリンクしている。樹種は、生物季節的に異なるステージにおいて生き残りを促進するため、それら栄養源のために競争し、N やP の取込・貯蔵・再利用のための異なるメカニズムを発達させている。一般的に、葉の老化の前に、N とP は葉から再吸収され、冬の間に土壌から吸収されたN とP と共に木質組織に貯蔵される。再吸収が終了すると、葉は器官脱離し、落ち葉は土壌のN とP の組成に影響する。そして春には、貯蔵されたN とP は芽の成長を支えるために移動する。しかし、それらは種依存であるため、それぞれの植物組織へのN およびP の再吸収の程度や、木全体の貯蔵への寄与を定量化する必要がある。植物のN とP の要求を満たすために、多くの樹種が寄宿植物に栄養を運んでくれる土壌菌類と共生しており、このような外生菌根菌が重要な役割を果たしている。しかし、多くの樹種において菌群落は同定されていない。菌を通して土壌からの栄養素の取り込みや、寄宿植物組織内の栄養素の移動を追跡することは、植物栄養素の循環を制御するメカニズムへの洞察をもたらすであろう。N サイクルを知るために、最も一般的な方法は、安定同位体分析（$\delta^{15}N$）である。しかし、N の実際の輸送はどのように起こっているのかいまだに分かっていない。遊離アミノ酸を定量することで、N の実際の輸送のメカニズムを明らかにし、N を輸送する成分の同定することが可能となった。一方で、P はタンパク質で輸送され、自然界に存在する1 つの安定同位体のもとでしか起こらないために、P の動きを追跡することは複雑であった。タンパク質に代わりに、P 含量を測定することは、経済的であり、樹種でのP サイクル解明に寄与するかもしれない。土壌の栄養状態、菌群集組成、土壌菌による栄養素の取込、再吸収、貯蔵、再移動など生物季節的変動に伴って、樹木全体レベルでの主要植物組織での種別の詳細な理解が、成木でのN とP サイクルを理解するため、森林管理を改善するために不可欠である。

材料と方法：我々は細い根（直径2 ㎜未満）、太い根（直径2 ㎜以上）、辺材、葉および落葉中の総および利用可能なN とP 含有量、N 同位体比およびアミノ酸含有量測定、土壌菌群集組成分析を日本の冷温地帯に見られる4 つの典型的な樹種：スギ（Cryptomeria japonica、D. Don、n=9）、カラマツ（Larix kaempferi、Sarg、n=8）、ブナ（Fagus crenata、Blume、n=9）、黒ニセアカシア（Robinia pseudoacacia、L 、n=5）を用いて分

析した。植物組織は、4 つの生物季節的段階（シュート生長、新緑、落葉前後のステージ）でサンプリングされ、実測値を樹木全体の値としてアップスケールした。

結果：すべての樹種がそれぞれの葉から、かなりの量の N と P を吸収したことを発見した。N は、落葉前のステージ中で主に太い根に、落葉後ステージ中で辺材に少量が貯蔵された。P は落葉後の期間で、黒アカシアでは辺材中に多くの量が貯蔵されていたが、他のすべての種では太い根および/または辺材にわずかな量が貯蔵されていた。全ての植物の成長は、N によって制限されていた。スギは、N と P の吸収と貯蔵によって明らかにされたように、他の種より栄養素利用可能率の変動に対して耐性があり、栄養保持する戦略を持っているようであった。根および辺材に加えて、この種では落葉後にも残っている葉に N を貯蔵しており、老葉に比べ、若葉で有意に高い N を含量していた。冬期に残っている葉における同位体組成の変化から、この樹種が全ての植物組織で土壌から吸収した N を貯蔵していることが分かった。さらに、遊離アミノ酸の移動から、N の内部移動を説明することができた。対照的に、P は落葉する葉と、残っている葉から再吸収されていたために、根および辺材に独占的に貯蔵されているようであった。これらのサンプリングプロットにおける菌群落は、外生菌根菌が存在しない多様な菌群集を特徴としていた。菌類分類群の 51％はいまだに未知であり、この樹種の栄養素循環に関する理解を深めるためには、土壌菌類の同定に焦点を当てた研究の必要性を強調された。

カラマツは、N と P の要求が最も少ない樹種で、N と P の利用効率が高かった。$\delta^{15}N$ が変化しなかったため N の移動を追跡することはできなかったが、遊離アミノ酸は N 輸送を明らかにするために有用であることが証明された。この種では、P 貯蔵期間中にかなりの土壌 P が取込されており、P サイクルの方が N サイクルよりも効率的であることが示された。この種は多様な菌群集および樹種の栄養摂取を支える豊富な外生菌根菌と関連していた。

ブナは、最も効率的に再利用が行われる植物組織において高い N および P 含有量を有していた。葉の N:P 比が落葉時に、N から P へ成長が限定される条件にシフトしたため、この種は P でなく、むしろ N で効率的であった。我々は、葉の老化中の $\delta^{15}N$ 分画によって、（1）葉の ^{15}N -欠乏 N の太い根への N 再吸収、続いて（2）辺材への ^{15}N -豊富 N の再吸収、および（3）太い根に貯蔵された土壌 ^{15}N -欠乏の N 吸収の 3 段階の窒素貯蔵を明らかにした。さらに、遊離アミノ酸量の変化は、植物組織における $\delta^{15}N$ 分画を部分的に説明した。この種は、菌種の豊富さ、多様性で最も低く、外生菌根菌への高い依存性を示し、環境の変化に対してより脆弱になることを示唆している。

黒ニセアカシアは、土壌からの N 吸収とは無関係で、すべての植物組織においてN、P が豊富であり、N、P の再吸収と効率性は最も低く、栄養搾取戦略を示していた。しかし、この種はシュートの成長段階では N が制限されていたが、この N の制限は新緑期および落葉前では N および P が共に制限される状態に徐々にシフトした。落葉の N：P 比から、この種が有意な量で N よりむしろ P を貯蔵することを示した。再吸収された葉の P は、根および辺材の貯蔵能力を超えたため、他の植物組織は追加の P 貯蔵として作用しなければならなかった。この種の菌群集は非常に均質で、多くのな腐栄養性を示した。

結論：本研究は、4 種の樹種に関して栄養サイクルの新たな洞察を明らかにし、菌群落分析と一緒に、4 つの生物季節的ステージに沿って木全体レベルでの N、P、δ^{15}N、遊離アミノ酸測定の組み合わせは、それぞれの樹種での栄養サイクルの理解を深めるために有効なアプローチであることを示した。遊離アミノ酸の動きと、N の安定同位体測定をリンクさせることで、樹種における植物内部の N の動きおよび輸送をより信頼性をもって追跡でき、生物季節的なステージの間で植物組織での安定同位体の変動を有意に示す優秀な方法を見つけ出せるかもしれない。樹木の成長を促進し、ゆえに大気からの炭素固定促進させるために、堆肥の最も最適なタイミングをデータから推定でき、それらは種と栄養依存性であった。スギは他の樹種よりもより効率的に炭素結合することから、大気中炭素を減らすためにスギを植樹することを、これらの結果に基づいて、我々は推奨する。黒ニセアカシアはスギと同等の炭素隔離能力を示したが、これらは外来種であり、かなりの多様性を減少させていることから、黒ニセアカシアの植樹は勧めない。さらに、スギは人為的影響や地球規模の変動による栄養供給の変化に対処するのに、最も適した種であるように思われた。次なる研究では、特にスギにおいて本研究で明らかになった多くの数の未知の菌種を同定し、共生に関してより深い理解を得るために、菌根菌群集構造と寄宿植物の内部栄養状態との相互座用を分析していくことを目標としている。

Acknowledgements

I would like to express my thanks to my supervisor, Larry Lopez, first for encouraging me to come to Japan for writing my Master thesis and then for convincing me to stay for PhD studies. He always supported me, gave me advice and we discussed endless hours about many, if not ALL the topics. Further, during my studies he gave me a worldwide network to explore and always helped me to get in contact with the right people to support me. Next, I want to thank all my co-authors, who hosted me and/or supported me with advice, namely Akira Oikawa, Luisella Celi, Eleonora Bonifacio, José Antonio Bonet, Carles Castaño, Josu G. Alday and Hiroko Kurokawa. In addition, I want to express my gratitude to our forest workers Arai-san and Izuka-san, keeping my research sites accessible throughout my studies. My special thanks goes to all the people who supported me in my field and lab work as well as in my private life in Japan and Europe: Enta-chan, Sayako-chan, Sho-san, Alex, Sara, Cho-kun, Cristina, and everyone I forget here. Further, without Mark, John, Misako, Jay and Abe-san, I would not have been able to complete the first half of my PhD studies, as they employed me and thus enabled me to cover my living and study costs. For the second half of my studies I want to thank the Higashi-Sakata Rotary Club for accepting me as a doctoral scholarship recipient allowing me to focus more on my studies by dropping some of the part time jobs. I want to thank especially my counsellor Saito-san as well as Kudou-san and Naoyuki-san!

Finally, I want to thank my family for respecting and supporting my decision to live in Japan although it surely was not easy for them. Also, a shout out to my friends back home who always supported me although I chose to live so far abroad. I appreciated every single of your visits and it gave me a feeling of being at home, as you are part of my family. However, the most important part of my family that I want to thank is that girl I met on my first day here in Japan (again, thanks to Larry for introducing us) and that I finally married. Without you, this adventure would have been impossible as you always kept supporting me even when I was close to insanity due to the sheer amount of work, Japanese bureaucracy, intercultural troubles and general stress. Thank you from the bottom of my heart Yukiko.