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Ecophysiological Study on the Natural Regeneration of the Two Larch Species with Special References to Soil Environment in Larch Forests

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Abstract

Larch plantations cover approximately 4700km^2 of the island of Hokkaido and have become the principle forest ecosystem in northern Japan. This study focused on how the environmental and biological factors affect the regeneration of Japanese larch (JL: *Larix kaempferi*) and hybrid larches F_1 (HL: *Larix gmelinii* x *L. kaempferi*) by comparing their ecophysiological characteristics of growth patterns (carbon balance, nutrient balance, the source of CO_2), especially relative to their root growth. Based on the review, we could forecast which larch species will have dominancy if they co-exist under the same environmental conditions in nature. In all, under relatively good growth conditions (for example full sunlight, 15°C soil temperature etc.), JL seedlings have a greater growth rate (allocate more biomass and nutrients) than that of HL. Thus, relative higher soil nutrient environment and above 7°C soil temperature are necessary for the growth of JL. Otherwise, HL will be more competitive than JL for example in cold regions or higher regions since HL will be better able to grow under a low or wide variation in soil temperature, or low nutrient soil conditions. Given the slightly shade conditionsuch as at a gap in the forest, or fertile soil condition, HL may also be dominate since the growth of JL was clearly suppressed under such situation. Moreover, HL allocated more photosynthates than JL under roots inoculated by ectomycorrhizae. But the ectomycorrhizae benefited the growth of both larch species. In this perspective, we should learn more about their interactions in regard to the establishment of regenerated larch seedlings and symbiotic microorganisms.

Key words: regeneration, Japanese larch, hybrid larch, light, soil temperature, fertilization, soil respiration, ectomycorrhizal symbiosis

Chapter 1
General Introduction

1.1 Japanese larch distribution

Larch forests essentially encircle the Northern hemisphere and within that approximately 20,000-km path, larch splits into 10 species and numerous varietiesofhybrids. These 10 species occupy a wild variety of ecological conditions and zones ranging from lowland boreal, to highmountains, to upper sub-Alpine conditions and extend southward to 25° latitude at high elevations and northward to 75° latitude in the boreal lowlands (Gower and Richard 1990). Deciduousness, intolerance to shade, efficient nitrogen and carbon use, and the ability to become established on poor soils enable the larch to become pioneer species in mixed-coniferous forests and the dominate species in the treeline (Gower and Richard 1990).

The Japanese larch (*Larix kaempferi*Sarg. = *Larix leptolepis* Gordon) is a species native to Japan. According to the forestry statistics of Japan, the artificial forest areas of Japanese larch are 1.09 million ha in size and account for about 11% of all total plantation forests (e.g. Bando 2013). The Japanese larch originates mainly in central Japan at a range of 1,100 to 2,700 m above sea-level (Asada and Satoo 1981, Asada and Sugawara 1983). Japanese larch forestation in Japan started in the early 1840s in the inland areas of highland region in central Japan. They are not indigenous to Hokkaido Island (Tatewaki *et al.* 1965). It was introduced into Hokkaido, the northernmost island of Japan, at the beginning of the 1900s (Sugimoto 1966, Bando 2013) for timber production due to its fast growth characteristics (Koike *et al.* 2000). Moreover, Japanese larch has also been planted in northeastern China, the Korean Peninsula and west Europe.

1.2 Importance of natural regeneration information of larch forests in northern Japan

Regeneration may be promoted by certain types of forest manipulation that can lead intentionally to new and more productive stages of forest growth. Due to its importance to forest management, the larch regeneration after exploitation has received particular attention (Bazzaz 1991). The regeneration consists of seed supply, the seedbed and the environment. All plants regenerate naturally after disturbance caused by fire, volcanic eruption, harvesting or other means, if given sufficient time. We usually want to rehabilitate disturbed areas promptly. Successful regeneration will only occur when all of these factors are favorable.

Why shouldwe focus on the natural regeneration of the Japanese larch forests? This is generally due to the advantages of natural regeneration. For examples,

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natural regeneration is less expensive than planting (i.e. less labor and heavy equipment are required). Furthermore, natural regeneration has no problem with the geographical origin of the seed, and the species and trees are well adapted to the site. New seedlings have better root system, or"natural" root morphology, than planted seedlings.

1.3 Statement of questions

1.3.1 Poor growth of larch stand

Recently, many poorly grown and damaged stands of larch have been seen in Hokkaido, Japan (Koike *et al.* 2000). These were planted in unsuitable environmental conditions such as poorly aerated and excessively wet soils, cold high-elevation areas, and in windyareas. Therefore, establishing and promoting the natural regeneration of larch forests for the purpose of conserving the natural environment has become an urgent topic of research. There are a number of issues regarding natural regeneration. However, the suitability of the forest environment and the unique requirements of the individual tree species have to be considered, as they are crucial for natural regeneration. Although present knowledge of ecological tolerance, interspecific competition, and siviculture practices are substantial, relatively little is known about the ecophysiological characteristics of the Japanese larch under changing environmental conditions.

The Japanese larch, which is recognized as a fast growing species, has been planted for timber production since the early 1900sin northern Japan (Sugimoto 1966, Bando 2013). However, it has been suffering from shoot blight disease and damages from grazing by voles. Therefore, the hybrid larches F_1 (*Larix gmelinii* var. *japonica* x *L. kaempferi*) was developed to resist these biological damages (Koike *et al.* 2000, Ryu *et al.* 2009). *L. gmelinii* was distributed in more northern areas through the eastern part of Eurasia (e.g. Schulze *et al.* 1995). We selected *L. gmelinii* originating from the Kril Ishlands as the maternal trees for breeding (Koike *et al*. 2000, Ryu *et al*. 2009). We are hoping for the successful natural regeneration of the Japanese larch and the hybrid larch in order to save labor cost and conserve the forestlands of northern Japan. The following questions are of concern.

1. Is there a significant relationship between the natural regeneration potential of larch and environmental factors?
2. How do the environmental factors affect the growth pattern (carbon balance, nutrient balance, the source of CO_2) of two larch species?
3. Which larch species will be dominant if they exist under the same environmental conditions?

1.3.2 Potential of soil for CO_2 source for regenerated larch seedlings and carbon sequestration

Regenerated seedlings use relatively high amounts of CO_2 near the ground (Bazzaz *et al*. 1987). In a mature forest, CO_2 fluctuation during daytime is very large (ranging between ca. 340 and 560 ppm= μ mol mol^{-1}) (Koike *et al.* 2001). Whatcauses this high CO_2 fluctuation in the frost floor from soil? How much does soil respiration rate fluctuate throughout the season? We should try to understand the soil respiration in forests as a CO_2 resource for naturally regenerated seedlings. Moreover, soil respiration is one of the most important components related tothe source and the sink of CO_2. Therefore, we should try to better understand the effect of environmental changes on soil respiration. The carbon stock rate in soils are estimated to be 50-75Pg C·$year^{-1}$ in terrestrial ecosystems worldwide and accounts for the second largest flux from terrestrial ecosystems, behind the gross primary production (100-120 Pg C·$year^{-1}$) (Raich and Schlesinger 1992). Forest soil respiration is the sum of heterotrophic (microbes, soil fauna) and autotrophic (root) respiration. Thus, the contribution of each group needs to be understood in order to evaluate implications of environmental changes on the CO_2 source for regenerated seedlings and soil carbon cycling and sequestration.

1.4 Research objectives and goals

It is anticipated that this research will provide information about the general status of the natural regeneration of the Japanese larch and its hybrid larch that could be used for planning, managing and conserving the larch forest on a sustainable base in northern Japan. For approaching the goal, the flame work of this study is as follows:

1. To develop a basic understanding of the factors that influence natural regeneration in larch forests.
2. To investigate the ecophysiological characteristics of two larch species, in particular the plasticity of the root system under varying environmental conditions.
3. To evaluate the contribution of soil respiration to net CO_2 flux from a larch forest as both CO_2 resources for seedling growth and a CO_2 sink.

The structure of this study is shown in Figure 1.1.

1.5 Approach for the goal

Two field sites were established *in situ* to investigate the natural regeneration conditions of the Japanese larch on Mt. Komagatake (42°04'N, 140°42'E, 1133m a.s.l.) in Hakodate, and soil CO_2 respiration in the Tomakomai National Forest (42°44'N, 141°31'E) of Hokkaido, Japan. According to the previous studies for natural regeneration of larch (e.g. Igarashi *et al.* 1987), environmental factors that influence seedling establishment is light condition, temperature, nutrient (Qu *et al.* 2003b) and the symbiosis with ectomycorrhiza (Yang *et al.* 1998, Qu *et al.* 2003a). Therefore these factors were testedsimultaneously with a greenhouse experiment in SapporoExperiment Forest of The Field Science center for Northern Biosphere, Hokkaido University (Figure 1.2).

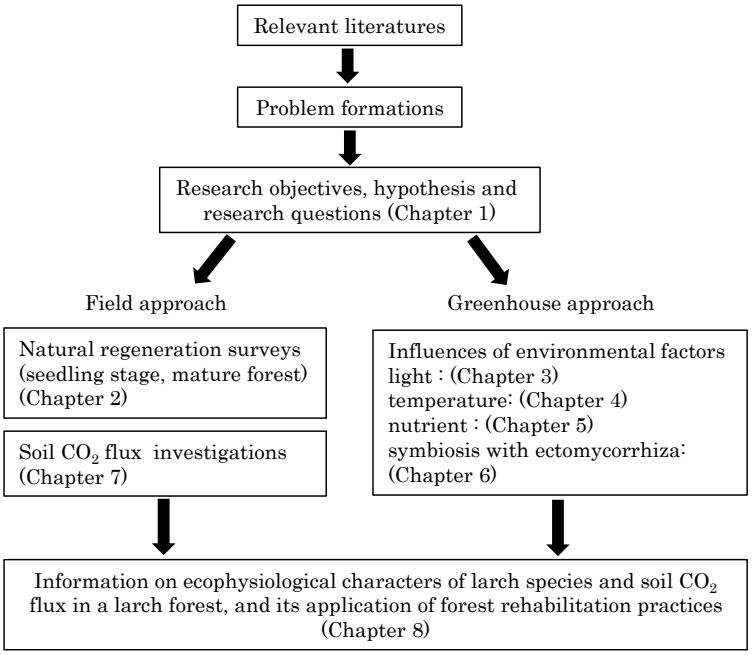

Figure 1.1 Structure of present study.

Figure 1.2 The two field study sites and greenhouse are located in Hokkaido. The closed cycles represent the field sites of Mt. Komagatake in Hakodate and Tomakomai National Forest on belonging to National Forest in Japan.

Chapter 2
Regeneration Surveys
2.1 Introduction

The successful natural regeneration of useful species has been pursed in order to save labor costs and conserve of forestland. However, not many species can regenerate successfully in natural condition because of high inter-specific and intra-specific competition within forests and in harsh environmental condition (e.g. Pickett and White 1985, Samejima 1985). If the physical conditions match the growth traits of plants, then the seeds and their seedlings can establish themselves under competitive conditions with other species (for example, microorganisms, plants, macro-fauna, etc.). The regeneration success of a tree species is strongly dependents on the micro-environment and the growth characteristics of the species. Therefore, it is generally accepted that we should know both the capacity of environment and the growth traits of plant species.

Larch trees regenerated fairly well under naturalcondition so as a result they have become dominant on disturbed sites following a volcanic eruption on Mt. Komagatakein Hokkaido (Sasaoka *et al*. 1997, Yajima 2000, Kayama *et al*. 2004, 2015). What kinds of environmental conditions are the keys to successful natural regeneration in larch species? It is expected that larch will establish itself under sunny, relatively warm soil (Qu *et al*. 2009), moderate-water conditions (Sasaoka *et al*. 2000), without any fungi destroying its seeds and seedlings. Moreover, this species is symbiotic with several kinds of ectomycorrhiza in Mt. Komagatake (Yang *et al*. 1998).

In order to clarify the natural regeneration and

invasion characteristics of Japanese larch, we investigated the micro-environment and growth responses of regenerated larch seedlings under different micro-site conditions. Firstly, we studied the effect of elevation on the growth of larch seedlings (on Mt. Komagatake). Next, we looked at factors of the forest stand structure affecting the seedling establishment (at Tomakomai National Forest).

2.2 Study sites and Methods

2.2.1. Site descriptions

Mt. Komagatake is an active volcano and located in the southwestern part of Hokkaido, northern Japan (42º04'N, 140º42'E, 1133m a.s.l.) (Photo 1). The eruption of 1929 produced ash, pumice and mudflows, which destroyed most of the vegetation on the slopes (Yoshii 1932). Pumice-flow eruptions have been recorded many times since 1640. Moreover, the recent major eruption was in 1929 (Yoshii 1942). The climate on Mt. Komagatake belongs to warm-cool temperate. Climatic data (50 yr mean) around the base of the volcano indicates a mean annual precipitation of 1138mm. Annual temperatures average 8.3°C, with a mean for January of –3.4°C; and for July 19.3°C (Yang *et al*. 1998).

The present ground layer vegetation is characterized by lichens and scattered shrubs such as *Salix reinii* and *Gaultheria miqueliana* (Kondo and Tsuyuzaki 1999). Plantations of Japanese larch were intensively established on the lower southwestern slopes of the mountain between 1953 and 1963. The larch is now more abundant towards the summit than any other native woody plants. Although, water conditions at the high elevation can be severe, photosynthesis of larch saplings are rarely inhibited by water stress due to their xylem water potential (Sasaoka *et al*. 2000, Yajima 2000, Kayama *et al*. 2015). We established three plots (1.5m x 5m) at both low (530 m a.s.l.) and high elevation (768 m a.s.l.).

The land of the Tomakomai region is also on a deep volcanic ash deposit (2 m in depth) of the last eruption of Mt. Tarumae in 1739. We selected an approximately 50-year old (as of 2000) Japanese larch plantation (500m×1500m) in Tomakomai National Forest (42°44'N, 141°31'E), Hokkaido, as a mature forest to be compared with Mt. Komagatake. The study was carried out in forest compartments of No. 1196 and 1198. The altitude is 115-140m a. s. l., and the average tree height was 18.5m in 2000. Most of the area is covered by volcanic soil. We created two monitoring sites at the forest floor in the larch plantation (with a density of 458 per hector, and a mean tree height of 24.3 m as of 2002) and its gaps (diameter was ca. 13m), which was formed as a result of the thinning treatment in 1993.

2.2.2. Monitor of microenvironment

On Mt. Komagatake, two auto-logged thermometers (Thermo Recorder mini, RT-30S, Espec Mic Corp. Osaka) were set up at both the high altitude (768m) near the tree line and relatively lower altitude (530m). Three thermometers were set to monitor the air temperature, temperature at soil surface and soil temperature at a 10cm depth from June2ndto October 9th, 2003. Thermo-recorders were also set up in the forest understory and gaps (ambient, soil surface at 1cm depth and 10 cm depths) in the Tomakomai National Forest from June 2nd to October 9th, 2003.

In order to investigate the growth status of plant during the growing season in 2003, 20 plots (1m x 1m) were set up at each altitude. The current year's shoot and total shoot height were measured on June 2nd, July 30th and October 9th, 2003. Twelve seedlings of naturally regenerated larch species, including surface soil and deeper soil (15–20cm depth) among the roots, were sampled from high and low altitudes on Mt. Komagatake, respectively. The infection ratio of ectomycorrhiza on roots (diameter<2mm) was evaluated using the following formula described by Beckjord *et al*. (1985) and Qu *et al*. (2004b):

$$PESR(\%) = ESR/(ESR+NSR) \times 100,$$

where ESR and NSR were the number of ectomycorrhizal and non-ectomycorrhizal short roots per plant, respectively.

After analyzing the ectomycorrhizal infection rates of rootsusing a microscope (Olympus, Tokyo), plants were separated into stems, branches, needles, dark roots and fine roots (diameter<2mm) and dried at 60°C for 48hr. Then, samples were milled and homogenized. Fresh soil was sampled from surface and deeper, sieved and the pH was measured by a pH meter (MP 220, TOA Electric Co., Ltd. Tokyo). Finally the soil was dried at 110°C for 48hr. The N concentration of each part of the plant was analyzed by the N-C analyzer (NC900, Shimadzu, Kyoto, Japan). The concentration of P was analyzed using an inductivity coupled plasma, ICP analyzer (IRIS, Jarrel ash, Franklin, MA, USA). The statistical tests were performed with the General Linear Model (GLM) of SAS (SAS Institute, Inc., 1996).

2.3 Results

2.3.1 Temperature fluctuations

From June to October, the maximumair temperature, surface soil temperature and deep soil temperature at 10cm depth on Mt. Komagatake was 28.5, 38.3 and 21.4°C at the high altitude, and 30.1, 52.2 and 23.3°C at a low altitude, respectively. An extremely high temperature of 52.2°C at the soil surface was instantaneously detected but it did not last along time. The minimum air temperature, surface soil temperature and soil temperature at 10cm depth on Mt. Komagatake was 1.0, 0.7 and 7.6°C at a high latitude and 2.4°C, 2.5°C and 8.6°C at a low altitude, respectively. The mean temperature of air, soil surface, and at a 10cm depth in soil were 14.1, 14.6 and 15.1°C at a high altitude and 15.6, 17.2 and 16.5°C at a low altitude on Mt. Komagatake (Table 2.1). The temperature variation of the surface soil was larger than that of the air temperature and the 10cm deep soil temperature (Table 2.1).

The maximum air temperature, and soil temperatures at 1cm and 10cm depths from June to October were

Table 2.1 Maxinum, minimum and mean air temperature, surface soil temperature and in soil temperature at a 10cm depth at high (768m) and low (530m) altitudes on Mt. Komagatake and in the gap and forest of the Tomakomai National Forest from June to October 2003. The values of mean temperature were the means and standard deviations (n = 1656)

			Maximum (°C)	Minimum (°C)	Mean (°C)
Mt. Komagatake	High altitude	Air temperature	28.5	1.0	14.1 ± 0.11
		Surface soil	38.3	0.7	14.6 ± 0.14
		10cm depth in soil	21.4	7.6	15.1 ± 0.06
	Low altitude	Air temperature	30.1	2.4	15.6 ± 0.11
		Surface soil	52.2	2.5	17.2 ± 0.17
		10cm depth in soil	23.3	8.6	16.5 ± 0.07
Tomakomai National Forest	Gap	Air temperature	34.9	-0.5	16.6 ± 0.13
		1cm depth in soil	25.0	7. 3	16. 8 ± 0.08
		10cm depth in soil	24.2	7. 8	16. 9 ± 0.07
	Forest	Air temperature	26.4	0.8	16.1 ± 0.10
		1cm depth in soil	26.4	0.8	15.3 ± 0.11
		10cm depth in soil	20. 9	9.7	16.3 ± 0.05

Table 2.2 The soil pH and infection rate (%) of ectomycorrhiza of Japanese larch seedlings at a high altitude (768m) and a low altitude (530m) on Mt. Komagatake. Values with the same letter (*a*, *b* and *c*) within column are not significantly different from each other at P<0.05 separated by the Least Squares Means using the GLM model of SAS analysis. All values are means and the standard deviations of six replicates

Locations	Plant condition	Infection rate of ectomycorrhiza (%)	Soil pH	
			Surface soil	Deeper soil (15-20cm)
High altitude	Good	72.1(6.51) ^{b}	5.63(0.06) ^{a}	5.73(0.04) ^{NS}
	Poor	51.1(5.05) ^{c}	5.54(0.01) ^{b}	5.74(0.14) ^{NS}
Low altitude	Good	89.6(2.49) ^{a}	5.64(0.06) ^{a}	5.70(0.13) ^{NS}
	Poor	56.2(1.29) ^{c}	5.63(0.03) ^{a}	5.71(0.01) ^{NS}

34.9, 25.0 and 24.2°C respectively. The mean air temperature and soil temperatures at 1cm and 10cm depths in the Tomakomai National Forest were 16.6, 16.8 and 16.9°C at the gap and 16.1, 15.3 and 16.3°C at the forest floor (Table 2.1).

2.3.2 Plant growth survey

The plant growth during the growing season (from June to October) and shoot height relationship at the low and high elevation sites are shown in Figure 2.1. When the current year's shoot height was above the regression line, the plants were defined as the "good-growth-plant". In contrast, the plants with a shoot height lower than the regression line were defined as "poor-growth-plant."

The growth rate of plants at the high altitude site was higher than at the low altitude site (Figure 2.1). However, there was no significant difference between the two altitudes.

2.3.3 Soil pH and ectomycorrhizal infection

The pH values of surface soil were lower than those of the deeper soil (about 15-20cm depth) regardless of the locations and plant growth conditions (Table 2.2). The pH of surface soil of the poor-growth-plant sites was significantly lower than the good-growth-plant sites at a high altitude. However, there was no significant difference at the value at a low altitude.

The infection ratio of ectomycorrhiza of roots of good-growth-plant was higher at the low altitude location than at the high on (Table 2.2). At both altitudes, the roots of good-growth-plants had a significantly higher infection ratio of ectomycorrhiza than that of the poor-growth-plants. Although the pH of surface soil at a high altitude was slightly lower (P<0.05), there was no difference in the infection rate of ectomycorrhiza (Table 2.2).

2.3.4 Nitrogen and phosphorus concentrations in larch plant

The plant nitrogen (N) concentration at the high elevation was higher than that of the low elevation. However, there was no significant difference between the two elevations. The N concentration of the needles was significantly higher in the good-growth-plant than in the poor-growth-plant at both altitudes and the N concentration of the stem and the fine root was also significantly higher in the good-growth-plant than in the poor-growth-plant at a low altitude (Figure 2.2). For the branches and the root organs, the N concentrations

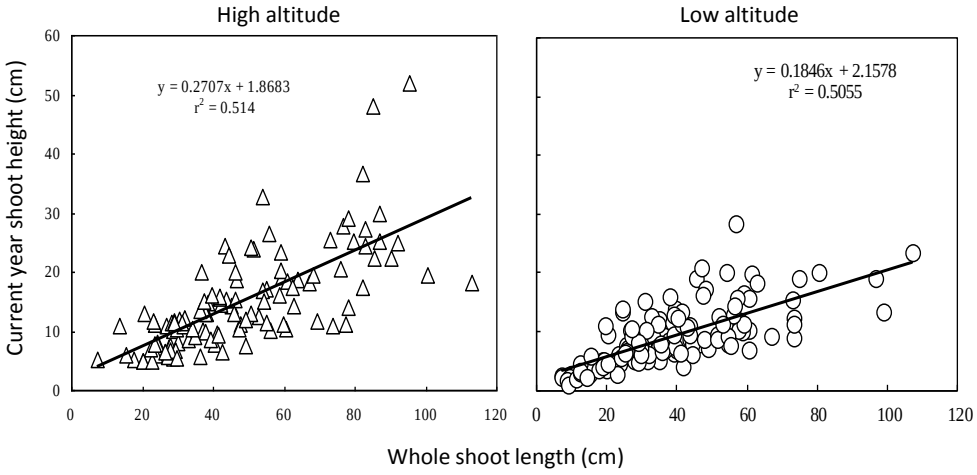

Figure 2.1 The relationship of height of current year shoot (cm) and whole shoot height (cm) at a high altitude and a low altitude on Mt. Komagatake.

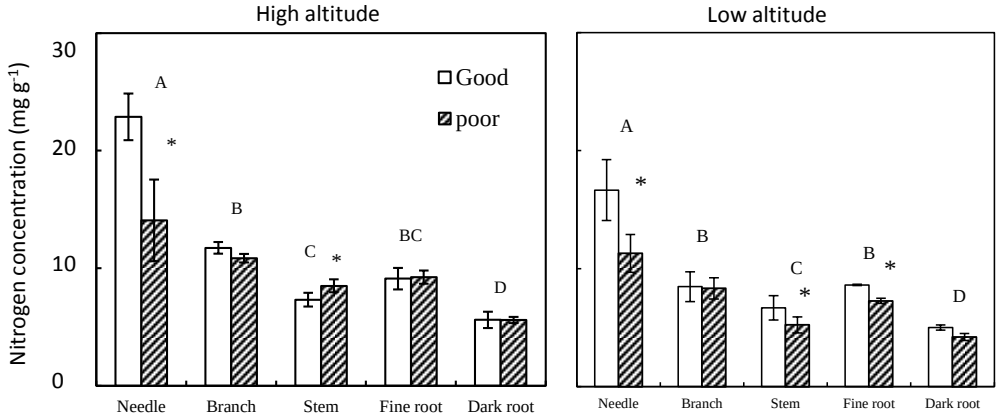

Figure 2.2 N concentration (mg g-1) of Japanese larch seedlings in the stem, branch, needle, dark root and fine root (<2mm) at a high altitude and a low altitude on Mt. Komagatake.

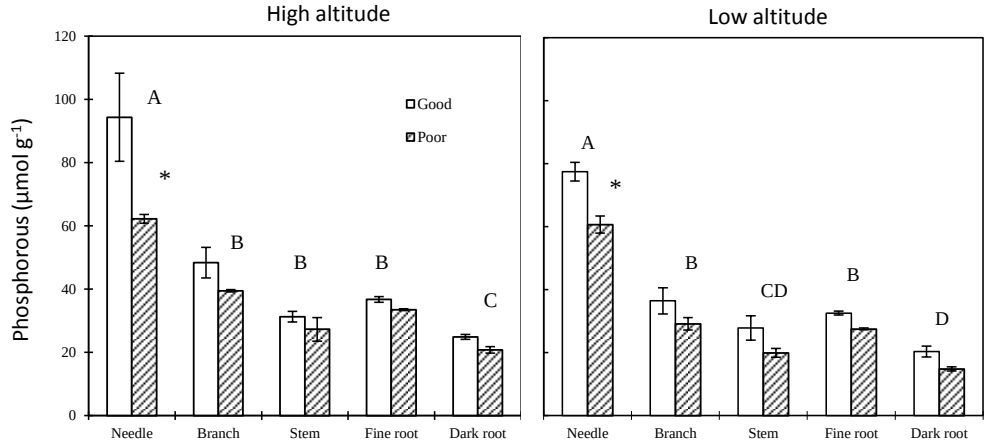

Figure 2.3 The phosphorus concentration (μmol per g) of Japanese larch seedlings in the needle, branch, stem, fine root (<2mm) and dark root at a high altitude and a low altitude on Mt. Komagatake.

did not significantly differ between the good-growth-plant and the poor-growth-plants at either the high and low altitude sites.

The phosphorus (P) concentration was higher in the needles in comparison to the branches, stems, fine roots and thick roots. For all the organs, P concentration was higher in the good-growth plants than it was in the poor-growth plants. Moreover, there were significant differences in the P concentration in needles of the good-growth and the poor-growth plants. However, there were no significant differences in the phosphorus concentration of plants at the high altitude and the low altitude sites (Figure 2.3).

2.4 Discussion

The larch plantation on Mt. Komagatake was established between 1953 and 1963. Larch trees generally grow fast and produce large amounts of seeds, which is well suited to colonizing at volcanic slopes and other areas after disturbances (Tatewaki *et al*. 1965, Koike *et al*. 2000). Kondo and Tsuyuzaki (1999) reported the distance from the seed source was the primary factor influencing the colonization of *L. kaempferi*, particularly in the early stages.

Soil temperature is an important factor affecting both the root and shoot growth of plants (Boucher *et al*. 2001, Kaspar and Bland 1992). Although our research period was limited, we showed that the fluctuation of temperatures was different on Mt. Komagatake compared to the Tomakomai National Forest. Moreover, the large fluctuation of soil surface temperatures may not benefit larch growth at the low altitude. However, in Tomakomai National Forest, the temperature at the gap was higher than that of the forest floor, which may accelerate the growth of regenerated larch seedlings.

In general, the leaf and shoot elongation is closely correlated with daily maximum temperature (Biscoe and Gallagher 1977). At high altitudes, the respiratory consumption of larch seedlings during the night may be suppressed. As a result, the shoots at a high altitude were longer than that of those at a low altitude even though the maximum photosynthetic rate at a high altitude is slightly lower as compared with lower sites (Kayama *et al*. 2004). In this study, we did not measure but the water condition is also an essential factor for the growth, survival and establishment of tree seedlings on Mt. Komagatake (Sasaoka *et al*. 2000).

In many respects, temporal variation in soil temperatures is qualitatively similar to the PFD (photon flux density) variation as reported by Bazzaz and Wayne (1994) in the deciduous broad-leavedforests in the northeastern US. Across the continuum, air temperatures are generally greater than surface soil temperatures at 1cm depth, which are higher than deep soil temperatures for the entire 24-hr period (e.g. Sipe 1990). Our results showed a similar temperature pattern in the forest and gap of Tomakomai National Forest (Figure 2.2).

The soil pH level is also considered to be an important factor for larch establishment in relation to the symbiosis of ectomycorrhizae formation. Activities of ectomycorrhizae are usually accelerated at slightly lower pH levels (Smith and Read 1997; Qu *et al*. 2010). However there was no close relationship between the pH and infection ratio of ectomycorrhiza in our study (Table 2.2). We only found large differences in the infection ratio that was affected by the growth (good vs. poor) of seedlings. Determining how soil pH influenced the ectomycorrhizae formation and growth on Mt. Komagatake still requires further study.

Yang *et al*. (1998) assessed the types, occurrence patterns and diversity of ectomycorrhizae in *L. kaempferi* seedlings along an elevation gradient on Mt. Komagatake. They considered that the lower infection ratio of ectomycorrhiza at a high elevation might be the result of less litter at high altitudes. Moreover, larch seedlings usually grow near shrubs such as *S.reinii* and *G. miqueliana*, especially at the high elevations (Kondo and Tsuyuzaki 1999). We also found ectomycorrhizae colonize with these kinds of ground layer plants. It may clarify that the nutrients and carbon translocation within the mycelial network.

The N concentration of plants at the high altitude was significantly higher than that at the low altitude. It may be an acclimation of adaptation for the larch at high elevations to keep a relatively high photosynthetic rate during the shorter growth period (Körner 1999, Larcher 2003). The N concentration of the needles differed significantly between the good-growth-plants and the poor-growth-plants. Moreover, the P concentration of the needles was significantly higher in good-growth-plants than it was in poor-growth-plants (Figure 2.3). This demonstrated that the N and P concentrations of the needles could be a key factor in influencing the growth of larch seedlings (Qu *et al*. 2003b).

The N and P concentration of needles and branches in good-growth-plants were higher at high altitudes than at low altitudes. This tendency is also found in alpine plants because plant size at high altitudes is small with a high concentration of nutrients (Körner 1999). At low altitudes, phosphates in the stem may be an essential storage location for growth. We must detect the seasonal trends of P and N in seedlings under field conditions to analyze different growth patterns.

Chapter 3
The Influence of Light Condition on Growth

3.1 Introduction

The level of irradiance is an important ecological factor for all photoautotrophic plants depend on it (Lambers *et al*. 1998). For example, light energy directly drives many fundamental plant and biophysical processes (photosynthesis, stomata conductance, transpiration, and leaf temperature). Light energy also indirectly influences many secondary plant processes, including plant growth, seedling regeneration, the vertical structure and crown shape of forest stands and the uptake and emission of trace gases that participate in biogeochemical cycling and atmospheric chemistry.

A remarkable feature of the photosynthetic apparatus of plants is its adaptability to wide range of light conditions. When a canopy gap forms, seedlings and understory plants receive high irradiances. Closure of the gap requires the seedlings to adjust to the low availability of light. Survival in the shaded understory demands maximization of light capture for photosynthesis concomitant while minimizing the loss of energy and carbon in respiration. By contrast, leaves exposed to a high light must be able to make efficient use of available energy while avoiding the possibility of a loss of photosynthates because of photo-inhibition or other environmental stresses (Qu *et al*. 2004a). The capacity to accomplish the balance is greatly influenced by changes in other environmental factors such as nutrient availability and temperature, which is often accompanied by changes in light availability.

Plants can acclimate to their light environment at

several integration levels. Firstly, they can change the fraction of biomass invested in leaves, stems and roots. Secondly, they are able to modulate the leaf area per unit biomass invested (in other words, specific leaf area: SLA) in leaves, by altering their anatomical structure. Thirdly, they can change the relative investment of nitrogen between photosynthetic components (e.g. Evans and Poorter 2001).

Larch is considered to be a light demanding deciduous conifer and under natural conditions regenerates fairly well and has become dominant on disturbed sites following a volcanic eruption on Mt. Komagatake of Hokkaido (Sasaoka *et al*. 1997, Yajima 2000). It is necessary to investigate the light acclimation capacity of two species of larch seedlings in order to understand the regeneration of the larch species on Hokkaido Island. Moreover, with the development of leaves on the canopy trees, the irradiance availability at the forest floor will be reduced. In general, nitrogen affects the photosynthetic capacity (Evens 1989a, b). The allocation pattern of nitrogen is strongly affected by light environment. Therefore, the combination effect of light and nitrogen level on young seedlings of the two larch species was examined. How do the larch species adapt their regeneration to a shade environment? Under different nutrient conditions, which species, Japanese larch or hybrid larch, will be dominant?

In this chapter, we discuss the role of the light environments on the growth of two species of larch seedlings under different fertile conditions in order to investigate the regeneration pattern of the two species.

3.2 Materials and methods

3.2.1 Experiment site and design

The experiment was conducted at the greenhouse, belonging to the Agriculture Faculty of Hokkaido University. The experiment was comprised of four light regimes providing reductions of 8%, 16%, and 32% of full sunlight as well as an unshaded, full sunlight control (100% rPPFD). Each light treatment consisted of two fertilization levels, 5mg and 25mg (low and high fertilizer treatment, respectively, referring to Qu *et al*. 2003b).

3.2.2 Plant materials and establishment

Seeds of Japanese larch and its hybrid (F1) were obtained from the Uryu Experimental Forest of Hokkaido University. After the seeds were put in 4°C for a 10-day cold treatment, they were individually germinated in pots with the soil media (clay-loam soil, peat moss and vermiculite (2:2:1 by volume)) for 30 days. Thirty-day-old healthy seedlings of the Japanese larch and the hybrid larch were fertilized using a mixed nutrient solution (N: P: K (5:10:5)) at weekly intervals. Sixty pots were placed outside on a uncovered table (100% relative photosynthetically active photon flux (rPPFD)) or covered with a black shade cloth (32% rPPFD, 16% rPPFD or 8% rPPFD, respectively). The maximum solar irradiance of the 100% rPPFD treatment on sunny days was about 1500μmol $m^{-2}s^{-1}$, comparable to the light conditions in an open canopy gap (Matsuki *et al*. 2003). The 32%, 16% and 8% (about 480μmol $m^{-2}s^{-1}$, 240μmol $m^{-2}s^{-1}$ and 120μmol $m^{-2}s^{-1}$) treatments simulated the light conditions of large, middle and small canopy gaps, respectively.

3.2.3 Harvest and chemical analysis

The shade treatments continued for three months in order to learn the growth and establishment processes of the two larch species. Eighty seedlings were harvested at four-week intervals. All harvested seedlings were separated into leaves, stems and roots. The root area and needle area were determined on fresh roots and needles using Area Meas (Hongu, Akinori MYKA. Lab.1.01 Ver, 1995). Finally, the parts were dried to a constant mass at 60° and weighed. Samples were milled and homogenized. Chlorophyll content was determined using the Dimethyl sulforxide (DMSO) method described by Shinano*et al*. (1996). The N concentrations of the needle, stem and root were analyzed using a N-C analyzer (NC900, Shimadzu, Kyoto, Japan).

3.2.4 Leaf gas exchange

CO_2 assimilation rates were measured with a Li-Cor LI-6400 photosynthesis system. The light-saturation rate of CO_2 assimilation was measured at an ambient CO_2concentration (at 360ppm). We preset the PPFD to 1500, 1000, 600, 300, 200, 100 and 0 μmol $m^{-2}s^{-1}$ with a Li-Cor 6400-02B red/blue light source fitted to the leaf cuvette. The CO_2 saturated photosynthetic rate was measured at a saturated PPFD of 1000μmol $m^{-2}s^{-1}$ to detect A-Ci curve (Sharkey 1985). The CO_2 concentration was controlled by the Li-Cor LI-6400 CO_2 injection system. We preset the CO_2 concentrations to 360, 100, 0, 360, 600, 1000, 1500ppm. All the measurements were made at ambient values of air temperature and relative humidity.

3.2.5 Statistical analysis

The mean values for dry mass of the organs, needle areas, nitrogen content, and SLA were analyzed using the nested procedure of SAS software (SAS Institute, Inc., 1996). The analysis of variance (ANOVA) and the general liner model (GLM) were used for balanced and unbalanced data, respectively.

3.3 Results

3.3.1 Growth response

By 12 weeks, the number of needles of the Japanese larch and hybrid larch had been markedly affected by irradiance availability (Figure 3.1). It decreased with a decrease in irradiance for all seedlings tested. However, the fertilized treatment did not show significant influences on the needle numbers within the same irradiance level. Compared to the control conditions, seedlings grown under shaded conditions showed a decrease in their needle areas. The lowest foliar area of seedlings was grown under 8% light levels. Fertilizer regimes didnot affect the needle area of either of the larch seedlings species (Figure 3.2).

The leaf area ratio (needle area per unit of dry mass) of seedlings grown in 100% light was significant lower

than that of seedlings grown in 8% light (Figure 3.3).

The total dry mass of the shaded seedlings decreased ($P<0.05$) compared to the control seedlings at the 4 weeks, 8 weeks and 12 weeks growing period for both larch species. The seedlings of the Japanese larch allocated more dry matter than those of the hybrid larch up to 12 weeks. There were significant differences for the larches at the second and at third harvest ($P<0.05$). However, there was no significant differencein dry mass allocation for both larch seedlings under low and high nitrogen level treatments (Table 3.1).

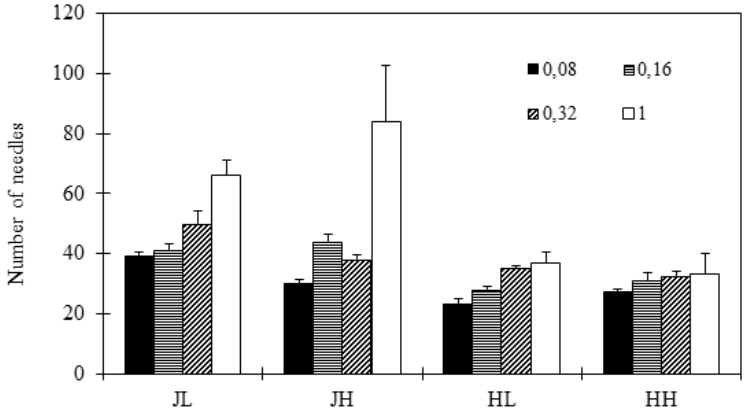

Figure 3.1
Needle numbers of Japanese larch and hybrid larch seedlings grown under four light regimes (8%, 16%, 32% and 100%) and two nutrient (5mg and 25mg) treatments.

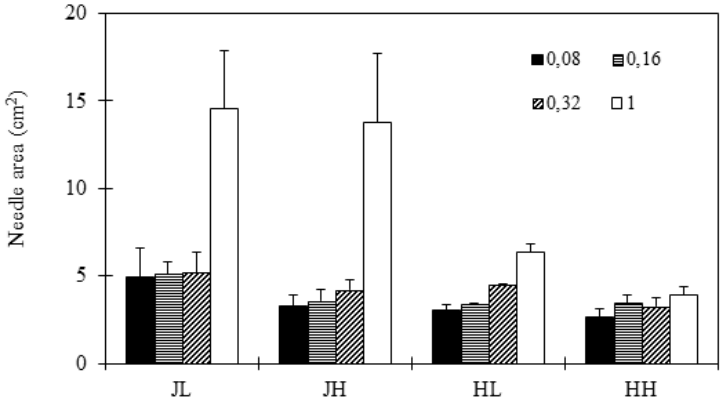

Figure 3.2
Needle area (cm^2) of Japanese larch and hybrid larch seedlings grown under four light regimes (8%, 16%, 32% and 100%) and two nutrient levels (5mg and 25mg) treatments.

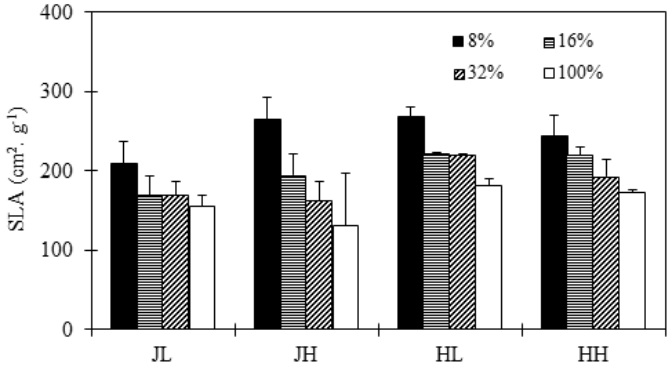

Figure 3.3
Specific leaf area ratio (SLA)(cm^2 g^{-1}) of Japanese larch and hybrid larch seedlings grown under four light regimes (8%, 16%, 32% and 100%) and two nutrient levels (5mg and 25mg) treatments.

Table 3.1 Effects of light and nutrient treatments on the dry mass allocation of the seedlings for the Japanese larch and hybrid larch during the growth period (12 weeks)

Nitrogen treatments		5mg				25mg			
Light regimes		8%	16%	32%	100%	8%	16%	32%	100%
Japanese	4 week	14.8 ± 1.7	17.0 ± 0.5	17.0 ± 3.7	19.1 ± 2.3	12.7 ± 0.8	13.1 ± 1.1	13.2 ± 0.5	21.1 ± 3.2
larch	8 week	43.7 ± 5.4	47.4 ± 2.5	57.0 ± 2.8	108.3 ± 10.1	19.1 ± 2.4	32.5 ± 4.7	43.0 ± 3.1	95.5 ± 9.3
	12 week	60.7 ± 5.6	69.4 ± 6.1	83.8 ± 8.3	150.2 ± 20.3	25.8 ± 1.5	54.7 ± 6.2	59.3 ± 2.7	241.2 ± 24.7
Hybrid	4 week	13.7 ± 3.7	12.6 ± 3.2	12.9 ± 0.9	18.6 ± 3.7	12.5 ± 1.1	11.3 ± 1.4	9.5 ± 3.2	22.1 ± 0.8
larch	8 week	28.1 ± 1.7	27.4 ± 1.5	38.4 ± 5.5	46.1 ± 6.3	25.4 ± 2.2	23.6 ± 2.7	29.4 ± 2.0	50.4 ± 1.8
	12 week	33.1 ± 4.4	32.5 ± 6.7	62.0 ± 5.7	77.4 ± 7.9	35.4 ± 3.5	32.6 ± 6.0	43.0 ± 4.6	83.6 ± 8.5

3.3.2 Partitioning of N

There was a significant difference in N content between fertilization treatments in the Japanese larch and the hybrid larch seedlings ($P<0.05$). The high fertilizer treatment resulted in a higher content of N in the needles, stem and root organs, compared to the low fertilizer treatment. There was no interacted effect between the fertilizer and irradiant treatment. The N contents of the Japanese larch and hybrid larch seedlings were influenced by shaded treatments. (Figure 3.7)

3.3.3 Chlorophyll analysis

Figure 3.2 summarizes the chlorophyll analysis completed at the 12-week. The total chlorophyll concentration per unit leaf area increased with increasing irradiance, with the high values of 0.33–0.38 in the 100% light and low values of 0.17–0.25 in the 8% light (Figure 3.5). The values of chlorophyll in the 32% and the 16% light treatments intermediate between those in the 100% and 8% light treatments. Similarly, the chlorophyll a/b ratios increased with increasing irradiance, from 8% to 100%, both in low and high nutrient levels (Figure 3.5).

3.3.4 Leaf CO_2 assimilation

By the 12th week, the steady-state rate of CO_2 assimilation increased asymptotically with increasing irradiance. Rates of CO_2 assimilation became highly light saturated at a lower PPFD in the shaded seedlings than in the 100% light seedlings (Figure 3.6). Seedlings grown in 100% light had a significantly higher light-saturated rate of net CO_2 assimilation (A_{max}) compared to other light treatments at the same fertilization level for both of the larch seedlings. Moreover, the maximum rate of carboxylation (V_{cmax}) had the same pattern as Amax. V_{cmax} was higher in the 100% light treatment than it was in the other three light treatments. However, Amax of the seedlings treated with a high N fertilizer was suppressed in shady conditions. This tendency was more clearin the Japanese larch seedlings (Table 3.2).

Figure 3.4 shows that the seedlings grown under 100% irradiance have the lowest shoot/root ratio compared with other three irradiance conditions under same fertilization levels. Moreover, fertilization increased shoot/root ratio in the four light regimes for both larches.

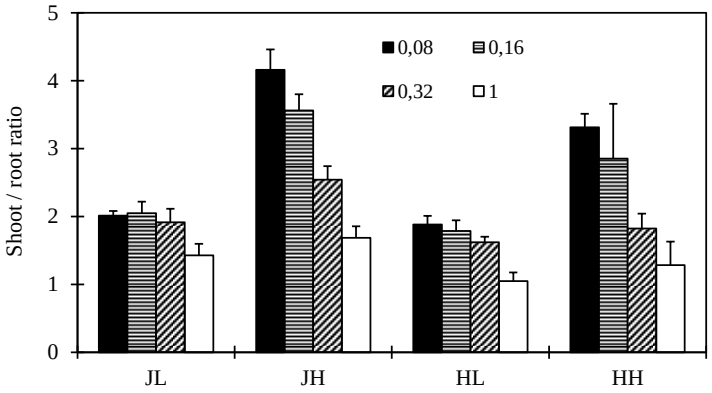

Figure 3.4 Shoot / root ratio of Japanese larch and hybrid larch seedlings grown under four light regimes (8%, 16%, 32% and 100%) and two nutrient (5mg and 25mg) treatments.

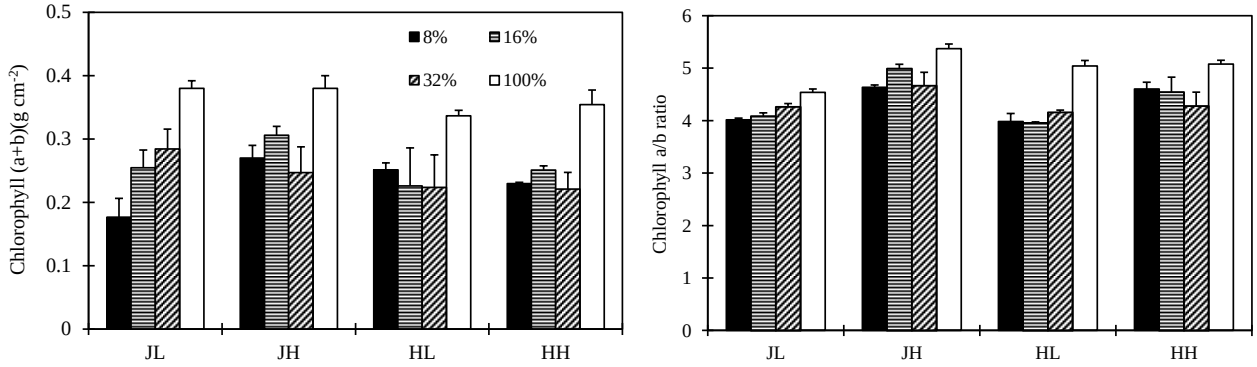

Figure 3.5 Chlorophyll (a+b) (g cm^{-2}) and chlorophyll a/b ratio of Japanese larch and hybrid larch seedlings grown under four light regimes (8%, 16%, 32% and 100%) and two nutrient (5mg and 25mg) treatments.

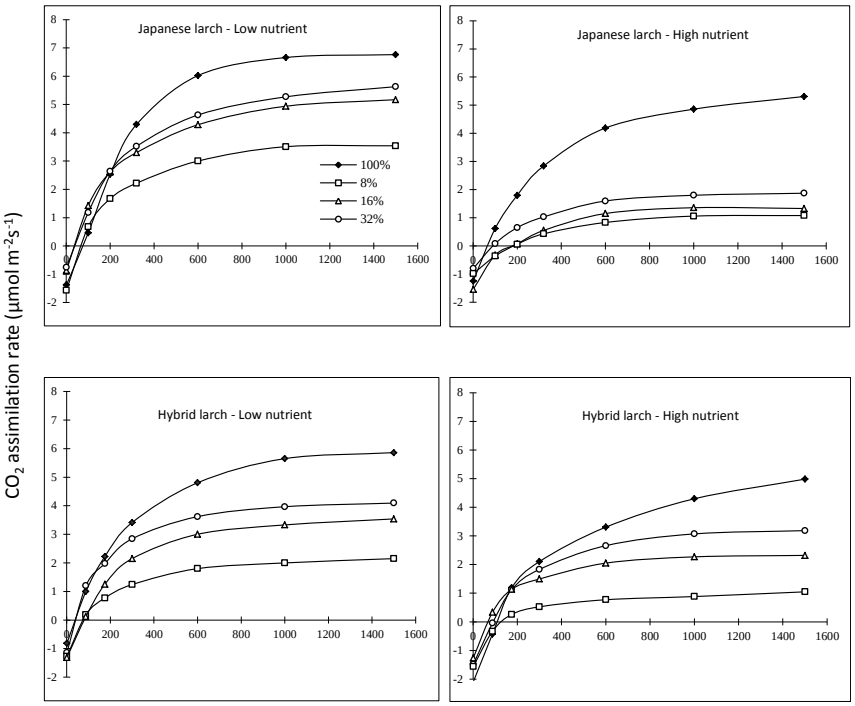

Figure 3.6 Relationship between leaf CO_2 assimilation rate and photosynthetic photo flux density (PPFD) in Japanese larch and hybrid larch grown under four light regimes (8%, 16%, 32% and 100%) and two nutrient (5mg and 25mg) treatments.

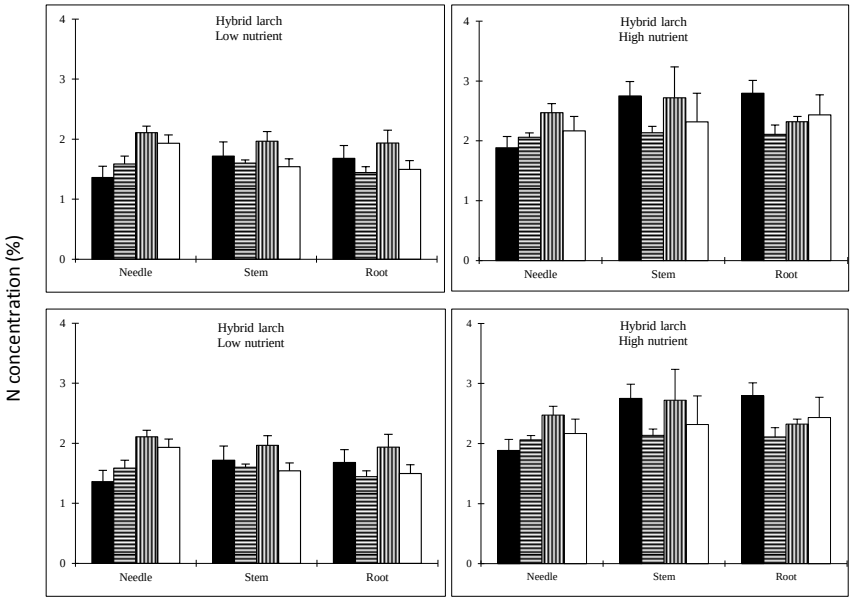

Figure 3.7 N concentration (%) in needle, stem and root of seedlings of Japanese larch and hybrid larch in four light regimes (8%, 16%, 32% and 100%) and two nutrient treatment (5mg and 25mg).

Table 3.2
The Mean Values of Light–saturated rate of net CO_2 assimilation at ambient P_a (A_{max}) and Maximum rate of carboxylation (V_{cmax}) under various light conditions (8%, 16%, 32% and 100%) and various fertilization levels (5mg and 25mg) of the Japanese and hybrid larch.* denotes a significant difference ($P<0.05$)

		Japanese larch		Hybrid larch	
		Nitrogen		Nitrogen	
rPPFD		5mg	25mg	5mg	25mg
A_{max}	8%	5.23	1.37	2.37	1.05
	16%	5.44	1.36	3.8	2.59
	32%	5.72	1.87	4.1	3.19
	100%	6.76*	5.72*	6.44*	4.98*
V_{cmax}	8%	31.8	21.7	36.8	25.1
	16%	34.2	20.8	24.8	30.2
	32%	39.1	29	41.8	38.7
	100%	63.2*	45.6*	50.8*	42.8*

3.4 Discussion

We found that CO_2 assimilation in the shaded seedlings tends to become more saturated at a lower PPFD and tends to have a lower A_{max} compared to the unshaded seedlings (Figure 3.6). This indicates that the larch seedlings had a low photosynthetic capacity under shady conditions. The reductions in growth and dry mass production were coincident with a reduction in CO_2 assimilation rates in shaded seedlings (Table 3.1 and Figure 3.6). The trend of a high CO_2 assimilation rate in the control plants and a low CO_2 assimilation rate of shaded plants was similar to that observed in previous studies (Matsuki *et al*. 2003, Senevirathna 2003).

Under the treatments of low nutrient supply, the Japanese larch tended to have a higher photosynthesis rate than that of the hybrid larch (not significant). However, under the high nutrient fertilization, the photosynthetic rate of the Japanese larch was clearly suppressed in the shaded treatments. Beside the pathway through the energy balance for N uptake, pH change by nitrogen supply (and coincidental NO_3-production) could be another reason. In contrast, this did not occur in the hybrid larch under the same treatments. Light energy directly drives many fundamental plant and biophysical processes. Plants need energy in order to uptake nutrients. However, rich nitrogen may sometimes become harmful to the growth of seedlings (Bazzaz 1996). In this study, it seems that this high nutrient supply had a negative effect on the growth of the Japanese larch in the shaded treatments. In general, plants need energy to assimilate nitrate from nitrogen (NO_3^- -N). Therefore, if the larches are able to assimilate NO_3^- -N, they need energy to reduce the nitrate nitrogen to the ammonium form of nitrogen (NH_4^+-N). However, the energy production of plants was suppressed under the shaded conditions. Thus, Japanese larch seedlings in shade could not use NO_{3-} -N. In this sense, the F1 may have a higher capacity to produce more energy used to assimilate NO_3^- -N and may adapt to this high nutrient condition better than the Japanese larch under low light conditions.

SLA was an important growth parameter in response to shade. The increase in SLA by shade indicated that thinner leaves of lower mass were produced under the shaded conditions (Figure 3.3). This is an adaptation of the larch seedlings under shaded conditions. In general, the thinner leaves can use the limited light more efficiently and gain the maximum carbon amount under the shaded conditions (Evans and Poorter 2001, Senevirathna *et al*. 2003). At the leaf level, a given amount of biomass can be spread over a small or large area. Plants grown in a high light generally have thick leaves with a low SLA, due to the possession of extra layers of palisade or longer palisade cells. This increases the number of chloroplasts and the amount of photosynthetic enzymes and thereby enhances the photosynthetic capacity per unit leaf area.

Shading increased the shoot/root ratio, which means an increased biomass fraction to the shoot and/or a decreased allocation to the roots (Figure 3.4). Such change in allocation maintains a constant transpiration rate per unit of root mass (Sims and Pearcy 1994) or may supply the greater demand for nutrients required for faster growth in high light.

The total chlorophyll concentration per unit leaf area and chlorophyll (Chl.) a/b ratio decreased in response to shaded treatments (Figure 3.5). Terashima and Hikosaka (1995) reported the Chl a/b ratio can be a useful indicator of N partitioning within a leaf, because this ratio should positively correlate with the ratio of PSII cores to the light harvesting chlorophyll-protein complex (LHCII). LHCII contains the majority of Chl b, and consequently it has a lower Chl a/b ratio (1.3–1.4) than other Chl binding proteins associated with PSII (Evans 1989; Green and Durnford 1996). Thus Chl a/b ratios should increase with an increasing level of irradiance at a given level of N availability (Hikosaka and Terashima 1995). The total leaf chlorophyll per unit leaf area decreased with shade, probably because the thinner leaves produce fewer cells per unit leaf area under shaded conditions (Senevirathna 2003).

In conclusion, the Japanese larch showed a more drastic change in response to decreasing PPFD due to a big change in the SLA. This tendency was more pronounced under a high nitrogen supply. However, the Japanese larch seedlings could not utilize the treated high nitrogen under shaded conditions. Based on these experimental results, the hybrid larch would be more dominant when it grows under a slightly shaded condition and in infertile soil conditions.

Chapter 4
The Influence of Soil Temperature on Growth

4.1 Introduction

Natural regeneration of Japanese larch species usually occurs at rocky moist sites or in mineral soils without litter and AO horizon (Igarashi *et al*. 1987), at soil temperatures between 1-30°C during the early growing season (Kitaoka, unpublished data). Plant growth is strongly regulated by environmental conditions, such as temperature (DeLucia 1986, Madsen and Brix 1997). Soil temperature is an essential factor affecting both root and shoot growth (Boucher 2001, Bowen 1991, Kaspar and Bland 1992, Qu *et al*. 2009). The optimum temperature for root growth of plants from temperate regions is between 10ºC and 30ºC, but growth may continue at around 0ºC (Lambers 1998). Even in the permafrost region in Siberia, regenerated seedlings of *L. gmelinii* began root development at around 5ºC (Korotkii *et al*. 2002, Prokushkin *et al*. 2002).

Since growth, development, and allocation are affected by temperature differently, temperature stress may induce imbalances in metabolic pathwaysin each individual. Soil temperature strongly affects the uptake of nutrients and absorption of water by roots. It is important to learn how soil temperatures affect root and shoot growth and the pattern of allocation to roots and needles of larch species. This is also relevant in view of the current rise in global temperature (Valentini *et al*. 2000). Research on the effect of soil temperature is therefore needed to learn how to improve resistance and tolerance to temperature extremes for the Japanese

larch and its hybrid.

When seedlings of both larches are able to germinate at same site, the two species compete. Which larch seedlings will dominate in forest gaps or at open sites where the soil temperature is subject to large fluctuations? If light conditions are suitable for the growth of larch seedlings, the temperature may be the main regulator of their growth and development. At a bare soil surface, the difference between the ambient temperature and the soil temperature is large. Temperature conditions at the root strongly regulate root growth (Fitter *et al*. 1998, Halter *et al*. 1997, Prokushkin *et al*. 2002, Wan 1999), photosynthesis (Strand *et al*. 2002), and the allocation of carbon between roots and shoots (Farrar 1988).

In view of the original distribution of the Dahurian larch, we hypothesize that the hybrid larch will acclimatize better to lower soil temperatures than the Japanese larch will.

4.2 Materials and methods

4.2.1 Plant materials and experimental design

Seeds of the Japanese larch and its hybrid larch were obtained from the Uryu Experimental Forest of Hokkaido University. Seeds were held at 4°C for 10 days as a cold treatment, and then were germinated in a soil medium (clay-loam soil, peat moss and vermiculite in volume ratio 2:2:1) for two weeks. In natural conditions, needle flush of the Japanese larch takes place approximately 10 days earlier than in the hybrid larch. Formation of terminal buds and needle coloration also begin two weeks earlier in the Japanese larch (Koike *et al*. 2000).

The experiment was performed using three root temperature regulators (ADVANTEC LF-680, Toyo Instruments Co., Chiba, Japan) in a growth room of a greenhouse belonging to the Field Science Center of Hokkaido University (43º W, 130º E).One seedling was planted in each rhizobox (about 287cm^3cavity volume, Eiken Instruments Co., Tokyo, Japan), which was wrapped with aluminum foil (Wu *et al*. 2002). Each plastic tray holds 40 rhizoboxes, and two plastic trays wereplaced in a water tank. The temperatures of the water tanks (7±0.2ºC, 15±0.2ºC and 2±50.2ºC) were controlled by a circling water system. The ambient temperature was 17–25°C, humidity was 40–60%, and the photoperiod was 16hr/day. The photoperiod was adjusted by fluorescence lamps designed for plant culture (FC4011 GL, Orderic Co., Tokyo, Japan) giving a photosynthetic photof lux density (PFD) of approximately 280µmol $m^{-2}s^{-1}$, simulating the value at forest edges (Kitaoka and Koike 2003).

4.2.2 Plant harvesting and measurements

The soil temperature treatments were maintained for 16 weeks. The seedlings were harvested at 4-week intervals. The photosynthetic rates of the seedlings were determined by a portable open-system IRGA (LI-6400, Li-Cor, Lincoln, NE, USA). Gas exchange was measured and the fresh needle area was also determined at that time. The light-saturated rate of assimilation of CO_2 at an ambient CO_2 concentration (360 ppm) was then calculated per unit needle area. All harvested seedlings were fractionated into leaves, stems and roots. The shoot heights were measured. The root area and needle area were determined by the fresh roots and needles with the Area Meas system (Hongu, Akinori MYKA. Lab.1.01 Ver, 1995).

Roots were scanned by taking a digital scanner, and root lengths were measured by a digital curvi-meter (Koizumi Sokki Mfg. Co., Ltd, Tokyo, Japan). Finally, the parts of the seedlings were then dried to constant mass at 60ºC and weighed. All samples were milled and homogenized. The N concentration in the shoots and roots was analyzed by the N-C analyzer. The total soluble sugar and starch content were determined using the modified phenol-sulfuric acid method and theperchloric acid method.

4.2.3 Statistical analysis

The positions of the rhizoboxes in the temperature regulator were changed each week to reduce edge effects. Statistical tests were performed using GLM routines from SAS (SAS Institute, Inc., 1996). Variables included the two species, the three temperature treatments and the four harvesting dates. Soil temperature was used as a grouping factor, and the measuring date was treated as a within-factor variable.

4.3 Results

The root biomass, root length and root areas of the two species (Figure 4.1) were significantly influenced by the soil temperature. On the other hand, the shoot dry mass, shoot height and needle areas of both species were also strongly affected by the soil temperature (Figures 4.3, 4.4). Growth increments of seedlings at soil temperatures of 15ºC and 25ºC were significantly greater than at 7ºC.

4.3.1 Growth response

A significant difference ($P<0.05$) was seen in shoot height increment between the Japanese larch and its hybrid larch (Figure 4.3). For the Japanese larch, the height of seedlings in the 7ºC treatment at 16 weeks was about 65% and 59% that of seedlings grown at 15ºC and 25ºC respectively; for the hybrid larch the figures are 72% and 65%. At the end of the growing season, the needle areas of the two larch seedlings were significantly different ($P<0.05$) (Figure 4.4). Japanese larch seedlings grown at 7ºC had smaller (4.98cm^2) needle areas than seedlings grown at 15ºC (12.63cm^2) and 25ºC (11.94cm^2), and the needle areas of hybrid larch seedlings were 3.32cm^2, 6.97cm^2 and 6.63cm^2 at respective soil temperatures of 7ºC , 15ºC and 25ºC . At soil temperature 7ºC, the total root length of the Japanese larch increased 3.8 fold during the entire growing season; at 15ºC the corresponding figures are 13.4 times and at 25ºC they are 15.2 times. In the hybrid larch the total root length increased by 2.5 times, 9.4 times and 11.9 times at soil temperatures of 7ºC, 15ºC and 25ºC respectively (Figure 4.1).

Root areasafter16th weeks did not differ significantly between the Japanese larch and the hybrid larch ($P>0.05$); see Figure 4.1. For theJapanese larch, the root

area at soil temperature 7ºC was 2.1 cm^2, differing significantly (P<0.001) from the value at 15ºC and at 25ºC. A similar trend was found in the hybrid larch, for which the root area at 7ºC was 1.5 cm^2, at 15ºC was 2.4 cm^2) and at 25ºC was 2.8 cm^2. At the end of the growing season, the RGR and NAR of seedlings grown at 7ºC were significantly lower than for seedlings grown at 15ºC and 25ºC (Figure 4.5).

4.3.2 Biomass

There was no significant difference in total plant dry mass between the Japanese larch and its hybrid larch, though the total plant dry mass of the Japanese larch was higher than for the hybrid larch,by 34.5%, 21.1% and 4.8% at soil temperatures 7ºC, 15ºC and 25ºC respectively (Figure 4.2). The total plant dry mass of both larch seedlings was significantly higher (P<0.05) at soil temperatures 15ºC and 25ºC than at 7ºC.

There was no significant difference in shoot dry mass between the Japanese larch and its hybrid larch (Figure 4.3). In the Japanese larch, the shoot dry mass was significantly higher (P<0.05) at 15ºC than at 7ºC and at25ºC. The hybrid larch had a similar peak, since the shoot dry mass was 46mg, 124.1mg and 119.3mg at 7ºC, 15ºC and 25ºC respectively. At 7ºC, the stem mass was lower than that of 15ºC and 25ºC in both larch seedlings, at the end of the growing season being 59.3% and 55.8% of the values at 15ºC and 25ºC in the Japanese larch, and 47.9% and 45.8% in the hybrid larch (data not shown). In the Japanese larch, the leaf dry mass was higher at 15ºC than that of 7ºC or 25ºC. However, there was no significant difference in needle dry mass between the 15ºC and 25ºC specimens of either larch species.

The root mass of the Japanese larch increased 5.6 fold from the initial root size at 7ºC, and for the hybrid larch the factor is 8.0 times (P<0.001) (Figure 4.2). At 15ºC the Japanese larch showed a factor 13.2 increase in root mass, and 21.4 times for the hybrid larch. At 25ºC the root mass of the Japanese larch had increased 12.3 times from its initial value, and 22.2 times in the hybrid larch.

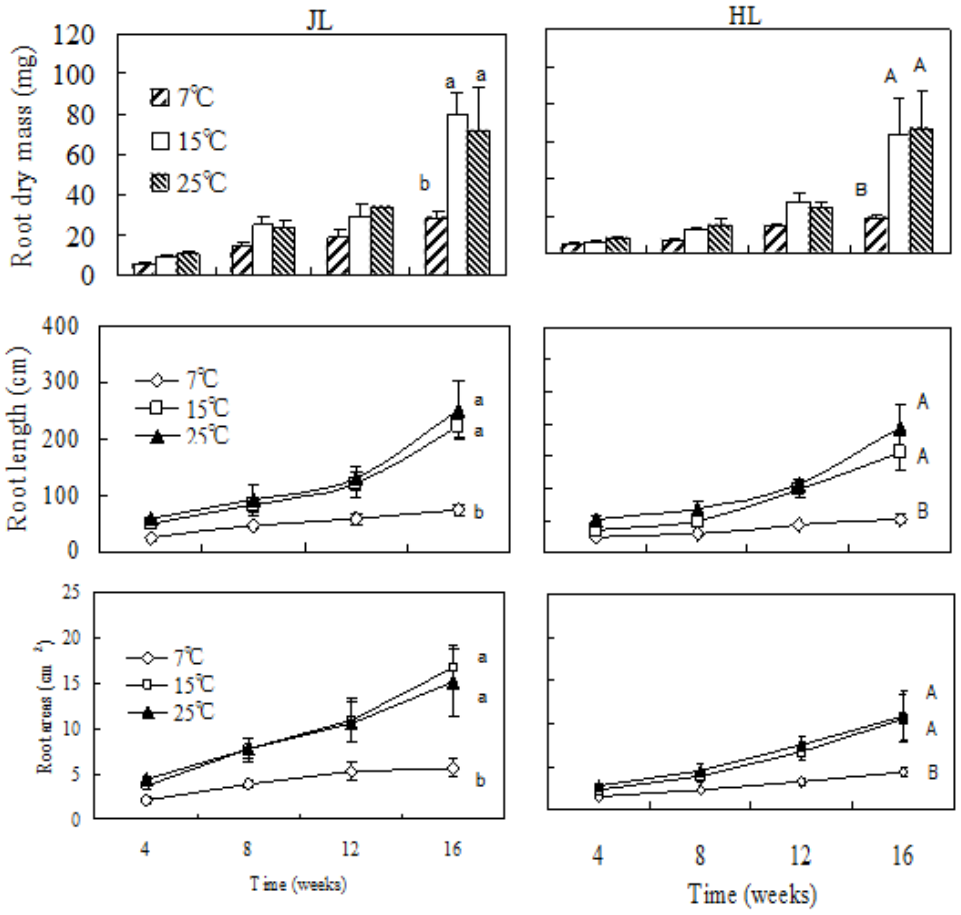

Figure 4.1 Root dry mass (mg), root length (cm) and root areas (cm^2) of seedlings grown at soil temperature of 7, 15 and 25ºC for 4, 8, 12 and 16 weeks. Mean of 9 seedlings ± SE. Mean of 9 seedlings ± SE. Data within a series followed by the same small letter do not differ significantly (P<0.05) in the Japanese larch and its hybrid larch, respectively.

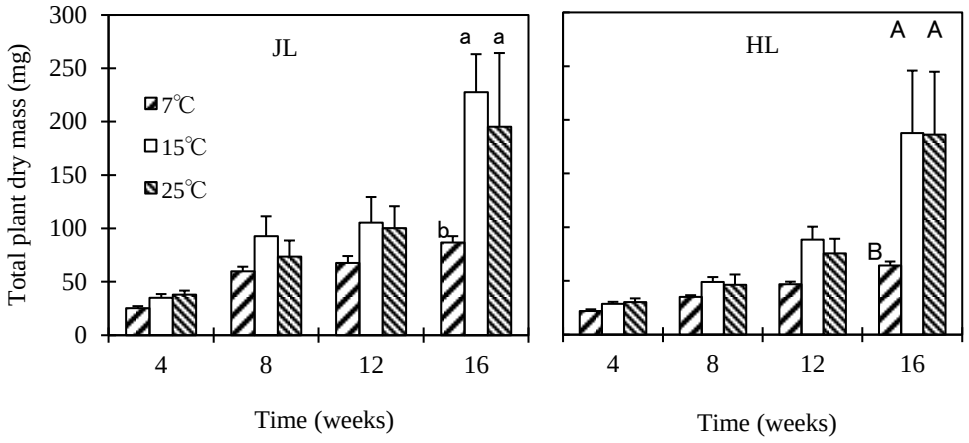

Figure 4.2 Total dry mass (mg) of seedlings grown at soil temperature of 7, 15 and 25 ºC for 4, 8, 12 and 16 weeks. Mean of 9 seedlings ± SE.

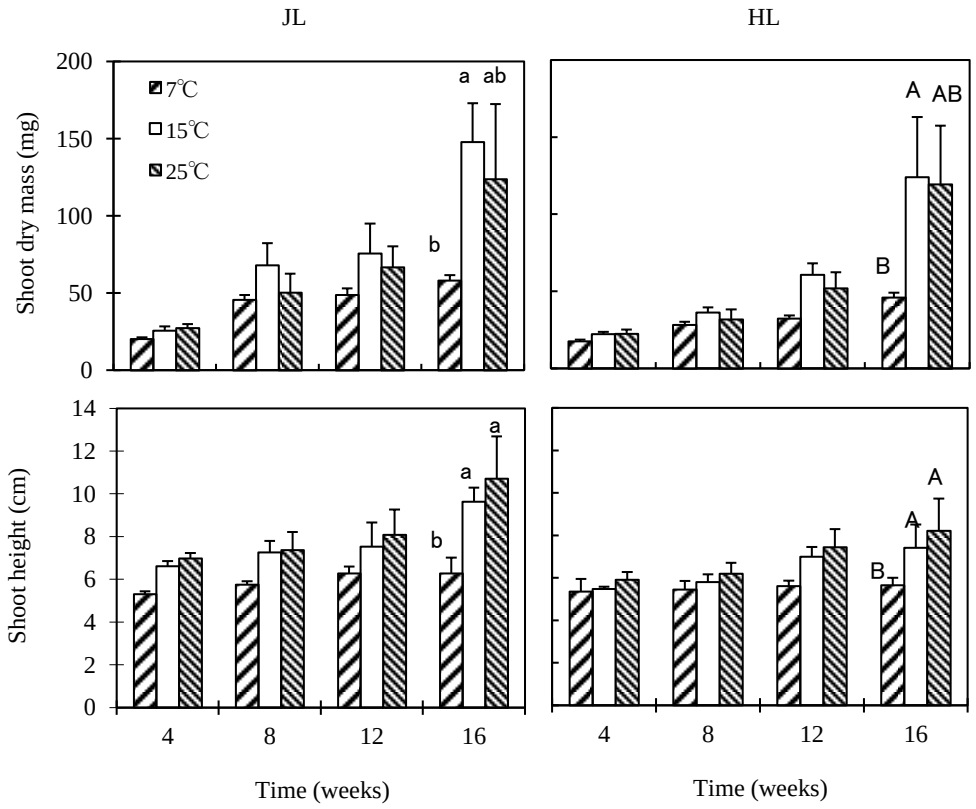

Figure 4.3 Shoot dry mass (mg) and shoot height (cm) of seedlings grown at soil temperature of 7, 15 and 25 ºC for 4, 8, 12 and 16 weeks. Mean of 9 seedlings ± SE. Data within a series followed by the same small letter do not differ significantly ($P<0.05$) in the Japanese larch and its hybrid larch, respectively.

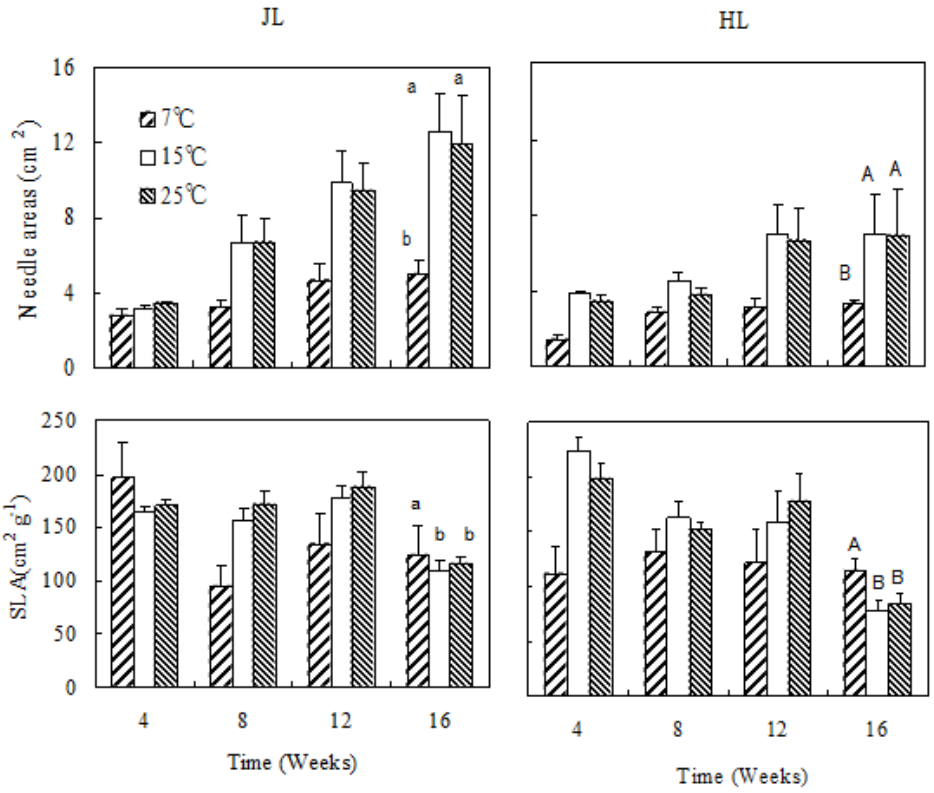

Figure 4.4 Needle areas (cm^2) and specific leaf area ($cm^2 \cdot g^{-1}$) of seedlings grown at soil temperature of 7, 15 and 25 ℃ for 4, 8, 12 and 16 weeks. Mean of 9 seedlings ± SE. Data within a series followed by the same small letter do not differ significantly ($P<0.05$) in the Japanese larch and its hybrid larch, respectively.

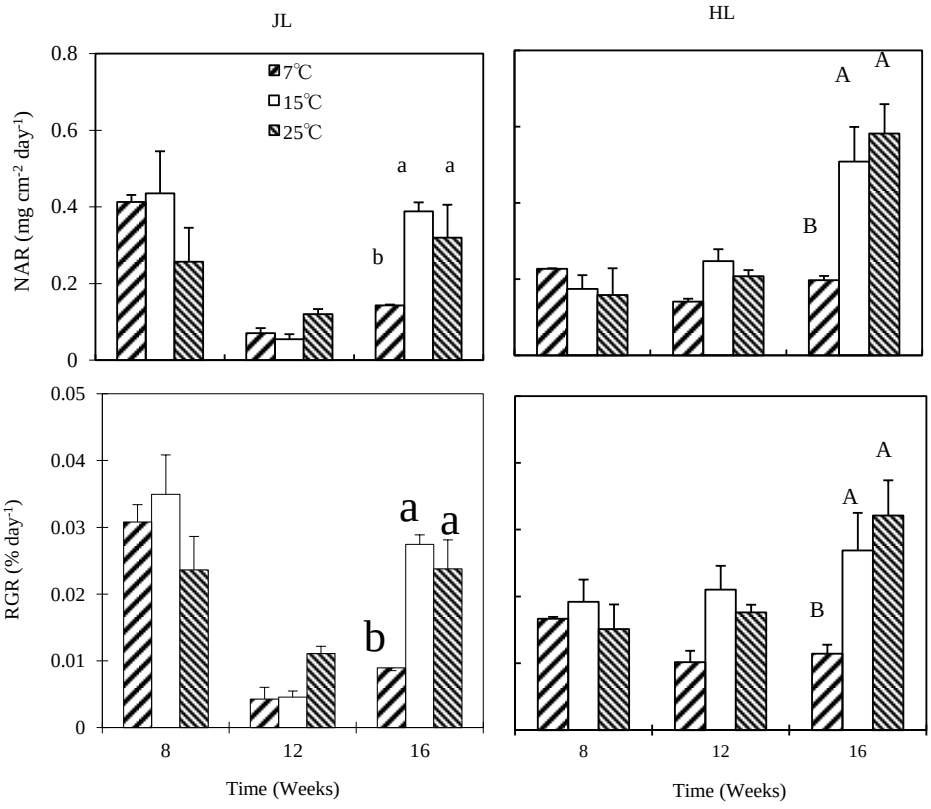

Figure 4.5 Relative growth rate (RGR) and net assimilation rate (NAR) of seedlings grown at soil temperature of 7, 15 and 25 ℃ from the 5-week to 16 weeks. Data within a series followed by the same small letter do not differ significantly ($P<0.05$) in the Japanese larch and its hybrid larch, respectively. Vertical bars represented the SE (n = 3).

Table 4.1 N content of needle, stem and root parts of Japanese larch (JL) and its hybrid larch (HL) seedlings at 7, 15 and 25 ºC soil temperatures. The values are means SE (n = 3). Different letters indicate significantly different means (P=0.05) in JL and HL, respectively. There are no significant different between JL and HL

Species	Soil temperature (°C)	Needle ($mg \cdot g^{-1}$)	Stem ($mg \cdot g^{-1}$)	Root ($mg \cdot g^{-1}$)
JL	7	10.0 ± 1.3B	6.3 ± 0.7a	10.0 ± 1.4C
	15	12.9 ± 1.2AB	4.4 ± 0.3b	10.8 ± 0.3C
	25	13.6 ± 0.2A	5.1 ± 0.1ab	8.4 ± 0.6C
HL	7	7.5 ± 1.4AB	5.5 ± 1.6B	15.8 ± 2.2C
	15	9.3 ± 1.4A	3.8 ± 0.7B	15.8 ± 1.3C
	25	7.1 ± 0.2B	3.4 ± 0.1B	10.1 ± 1.4C

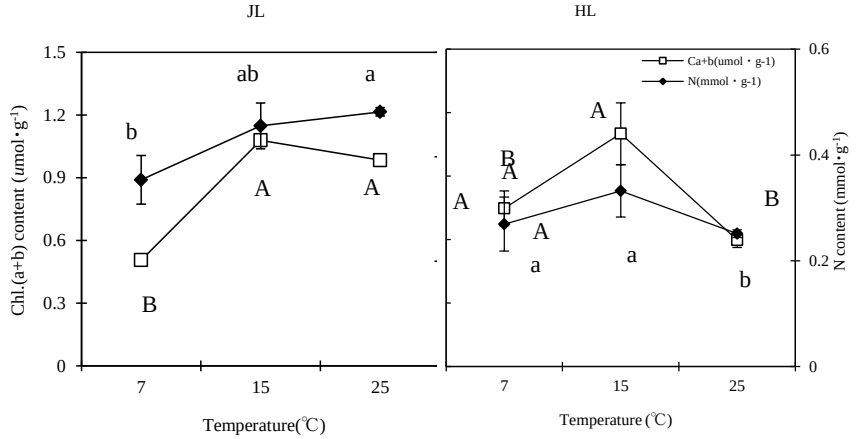

Figure 4.6 The relationships between N content (mmol・g-1) and chlorophyll a+b (Chl.a+b) (μmol・g-1) of Japanese larch and its hybrid larch seedlings grown at soil temperature of 7, 15 and 25 °C for 16 weeks. Vertical bars represent SE (n = 3 for N content and 9 for Chl.a+b). Data within a series followed by the same large capital or small capital do not differ significantly (P<0.05) in the Japanese larch and its hybrid larch, respectively.

4.3.3 Nitrogen concentration and chlorophyll

There were no significant differences (P>0.05) in the N content of seedlings between the Japanese larch and hybrid larch. Japanese larch seedlings showed significant difference (P<0.05) between the N content of the needle and stem (Table 4.1). The N content of the needle at 25ºC was significantly higher than at 7ºC. Seedlings grown at 7ºC had significantly higher N content than that of those grown at 15ºC. However, there was no significant difference in root N content between the three soil temperatures. The hybrid larch seedlings also showed significant differences in the N contents of the needles, stem and roots. However, there was no significant difference within needle, stem and root, respectively (Table 4.1).

At the end of the growing season, the chlorophyll content of the 7ºC specimens was lowest than that of those in the 15ºC and 25ºC specimens (Figure 4.6). In the Japanese larch, the chlorophyll content of seedlings at 7ºC was significantly lower than at 15ºC and 25ºC (Figure 4.6). In the hybrid larch, the chlorophyll content of seedlings at 7ºC differs significantly from seedlings at 15ºC, but not from seedlings at 25ºC (Figure 4.6).

4.3.4 Photosynthesis and PNUE

Amax was higher at 15ºC soil than that of 7ºC or 25ºC (Figure 4.7). At 7ºC, Amax of seedlings of both larches was significantly lower than at 15 °C and 25ºC (Figure 4.7). Amax increased with needle nitrogen concentration except in the hybrid larches at 25ºC.

The PNUE (photosynthetic nitrogen-use efficiency) had different patterns in the Japanese larch and the hybrid larch (Figure 4.7). The PNUE of seedlings was greatest at 15ºC in the Japanese larch, but at 25ºC in the hybrid larch.

4.3.5 Soluble sugar and starch concentration

The concentration of soluble sugar in Japanese larch seedlings grown at 7ºC was 9.1% of the dry mass; this is higher than the values of 5.0% at 15ºCand 4.6% at 25ºC (P<0.001) (Table 4.2). The concentration of soluble sugar in hybrid larch seedlings, in contrast to Japanese larch, showed no significant differences (P>0.05) with soil temperature; in seedlings grown at 7ºC , 15ºC and 25ºC the concentrations were respectively 10.2%, 10.5% and 10.1%. However, the concentrations of soluble sugar differed significantly

Table 4.2 Concentration of soluble sugar and starch (% of total dry mass) in shoots and roots of seedlings grown at soil temperature of 7, 15 and 25 ºC for 16 weeks (mean SE, n = 3). JL represented Japanese larch and HL represented its hybrid larch

Species	Soil temperature	Sugar (%)			Starch (%)		
	(℃)	Shoot	Root	Plant	Shoot	Root	Plant
JL	7	9.4 ± 0.3a	8.6 ± 1.1a	9.1 ± 0.7a	16.6 ± 1.7B	16.4 ± 0.7A	16. ± 61.2A
	15	5.3 ± 0.1b	4.4 ± 0.4b	5.0 ± 0.2b	22.4 ± 2.9A	10.2 ± 1.1B	18.1 ± 2.4A
	25	4.9 ± 0.9b	4.2 ± 0.9b	4.6 ± 0.9b	11.5 ± 0.6C	11.4 ± 1.0B	11.5 ± 0.7B
HL	7	10.9 ± 2.0A	8.7 ± 0.8A	10.2 ± 1.6A	13.6 ± 1.2A	16.7 ± 0.6A	14.5 ± 1.0A
	15	10.9 ± 1.1A	6.8 ± 1.0A	10.5 ± 1.0A	12.5 ± 0.6A	8.8 ± 1.1B	11.5 ± 0.8B
	25	11.5 ± 0.4A	7.2 ± 0.5A	10.1 ± 0.5A	14.2 ± 1.0A	9.7 ± 0.6B	12.6 ± 0.9B

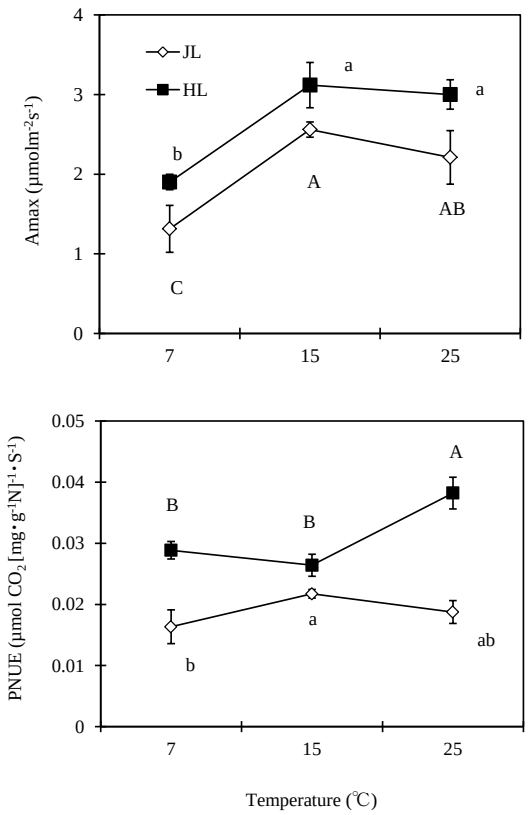

Figure 4.7
Comparisons of Amax and PNUE of Japanese larch and hybrid larch seedlings grown at 7, 15 and 25 ºC soil temperature after 16 weeks culturing. JL represented Japanese larch and HL represented its hybrid larch. Vertical bars represented the SE (n = 3).

(P<0.05) between shoots and roots for both species at all three temperatures. The Japanese larch and hybrid larch seedlings showed a significantly higher starch concentration in roots at 7ºC soil temperature than at 15ºC and 25ºC (Table 4.2).

4.4 Discussion

The soil temperature had clear effects on both Japanese larch and the hybrid larch. At a soil temperature of 7ºC, the root areas and root length of both larch seedlings were significantly lower than at 15ºC and 25ºC. Exposure to a low soil temperature clearly reduced root extension (Domisch *et al*. 2001, Fitter *et al*. 1999, Halter *et al*. 1997). It is well known that lower soil temperature inhibits the metabolic activity (cell division and growth hormone synthesis) of roots and reduces root water absorption. The reduced water absorption by roots therefore has an effect on photosynthesis through the water status. Boucher (2001) reported that the efficiency of which light that is captured by the shoot generates shoot growth and is partly controlled by soil temperature. It may be possible that trees exposed to relatively high air temperatures and cold soils are unable to conduct water fast enough to meet the transpiration demands of shoots (Wan *et al*. 1999). The slower shoot growth at 7ºC shows that a lower soil temperature reduces photosynthesis through the rate of carbon allocation (Korotkii *et al*. 2002, Larcher 2003).

The chlorophyll content, NAR and RGR in seedlings at 7ºC show significant differences from values at 15ºC in both larch species (Figures 4.5, 4.6). These results indicate that lower soil temperatures strongly influence not only root growth but also shoot growth in seedlings of the two larches.

The N content of needle, stem and root all differed significantly between the Japanese larch and the hybrid larch (Table 4.1). Needles and roots have higher N than stems at all three soil temperatures, which may be due to the higher activity in needles and roots than in the stem. It appears that roots of some species can successfully acclimatize to low temperatures and continue to be active when the soil temperature is only a few degrees above freezing. In such conditions, resources from photosynthesis become vital (Fitter 1998). A soil temperature of 7ºC caused smaller needle areas, with consequences for photosynthetic activity and the translocation capacity of photosynthates.

Larches occur in cold temperate and boreal climates in the northern hemisphere. In the Japanese larch and its hybrid larch, roots can successfully acclimatize to a soil temperature of 7ºC as found in *L. gmelinii* (Korotkii *et al*. 2002). The roots have good capability to acclimatize to suboptimal temperatures, and roots

acclimatized to low temperature contained 9-15 times more soluble carbohydrates (mostly sucrose and fructose) than controls held at 20ºC (Clarkson *et al*. 1974).

As plants acclimatize to the cold, cells show a characteristic increase in solutes, including soluble sugars and other osmolytes. Hare (1998) reported that a higher soluble sugar content leads to a reduced osmotic potential, which in turn favours water uptake by roots and retention by shoots. Acclimatized Japanese larch accumulated much more soluble sugar at 7ºC in shoots and roots (about twice as much as at 15ºC and 25ºC); see Table 4.2.

In the hybrid larch, however, the soluble sugar concentration in roots was highest at 7ºC, but not significantly different from values at 15ºC and 25ºC. At 7ºC, both the Japanese and hybrid larch seedlings showed a significantly higher starch concentration in roots than at 15ºC and 25ºC (Table 4.2).The increase in soluble carbohydrates with acclimatization is recognized as a cryoprotective measure for protecting cell membranes and organelles. The Japanese larch is apparently more sensitive to suboptimal low soil temperatures than the hybrid larch. This may be due to the different genotype of the two larches, since the Japanese larch is distributed naturally at lower latitudes i.e. central Japan (33° N, 134.5° E) than the Dahurian larch, which is the maternal plant of the hybrid larch.

With increasing soil temperature, the biomass of the shoots and roots of the two larch seedlings increases markedly. Lyr (1996) has reported that the optimum growth (total dry mass increment) root temperature of *L.decidua* is about 15ºC. Since the distribution of *L. decidua* and *L. kaempferi* is similar temperature regime, 25ºC is a high soil temperature for the Japanese larch. The highest soil temperature in northern Japan was about 25ºC at 5cm depth in 2000-2002 during the growing season (Qu *et al*. 2003b). A soil temperature of 25ºCincreased root length, but did not increase the root area or root biomass. Except for the shoot height of seedlings at 25ºC, the needle, needle biomass and shoot biomass were lower at 25ºC than at 15ºC. A temperature of 25ºC promoted shoot and root elongation but did not increase the biomass of seedlings of either larch compared to values at 15ºC.

Root temperatures that enhance biomass allocation to roots probably also increase the proportion of carbon required for root respiration. The concentrations of soluble carbohydrates in seedlings raised at 15ºC and 25ºC are lower than concentrations at 7ºC. This may be due to the greater respiration at higher soil temperatures. It has been suggested that an increase in root temperature may increase the demand for respiratory substrates in roots, resulting in lower carbohydrate concentrations in the entire plant or the shoot (Fitter 1998). The reason may be that the proportion of carbohydrates translocated to roots that is used in respiration, rather than in root biomass accumulation. A soil temperature of 15ºC accelerated growth of seedlings of the two larch species better than a temperature of 25ºC. Neither species prefers such high soil temperatures.

The content of N in the Japanese larch was greater than in the hybrid larch, especially in the needle (Table 4.1). Generally, a higher nitrogen content is associated with higher maximum photosynthesis rates (Field and Mooney 1986, Poorter and Evans 1998, Reich *et al*. 1995). However, the hybrid larch showed a higher Amax value than the Japanese larch, possibly due to the lower SLA of the hybrid larch. The SLA is a morphological factor since it is determinedtheby leaf dry matter concentration and leaf thickness. At the end of the growing season, the needle area was significantly lower in the hybrid larch than in the Japanese larch, due possibly to its thicker needle morphology. Moreover, the PNUE of the Japanese larch at a soil temperature of 15ºC was higher than that of 7ºC and 25ºC. The higher PNUE at 15ºC may be due to a high accumulation of light, i.e. of chlorophyll (Figure 4.6). The highest PNUE at 25ºC was in the hybrid larch, which may be due to differing amounts of respiration in the light (Poorter and Evans 1998).

The Dahurian larch is more resistant to low temperatures than the Japanese larch according to their natural ranges of distribution. The greater tolerance of low temperature by the hybrid larch than the Japanese larchmay be due to its ancestry inherited from the Dahurian larch. The hybrid larch will therefore be more competitive than the Japanese larch in cold regions (such as Northern Japan) and be better able to grow under wide variations of soil temperature.

Chapter 5
Influence of Nutrient on Growth
5.1 Introduction

In natural environments, the larch typically behaves as an early successional species. However, for post-forest fires, the soil fertility of bare land, after harvesting, erosion and landslide is very poor. Nevertheless, the larch usually invades such infertile conditions under full sunlight (Koike *et al*. 2000).Nutrient management in nurseries is essential for producing larch seedlings where the soil is usually fertile. Therefore, it is necessary to know the nutrient regimes for the growth habitat of larch species.

Root growth and development are strongly influenced by the growing conditions, such as soil fertility and the degree of moisture. Fine root development is usually accelerated by xeric and poor nutrient conditions (e.g. Fitter 1999). The development of the plant root system is exceptionally plastic, in comparison with almost all other plant organ systems (Fitter *et al*. 1998). The effects of soil nutrient availability on root growth, physiology, dry mass accumulation, or nutrient dynamics are often investigated by experimentally manipulating nutrient supply. Several approaches have been used including bulk fertilization, stationary culture and supplying nutrients exponentially to solid media (Miller and Timmer 1994, Timmer and Munson 1991).

Conventional fertilization is adding nutrition at the same concentration of fertilizer throughout the growing season. Irrespective of plant development stage with this method, initial nutrient supply usually exceeds

seedling demand due to it being a smaller size. In contrast, exponential fertilization technique can match nutrient supply with the seedling's growth and nutrient uptake during the exponential growth phase (Quoreshi and Timmer 1998, Quoreshi and Timmer 2000). In response to exponential nutrient supply, seedlings exhibit steady-state nutrition or undiluted concentration during the exponential growth phase (Timmer 1997), which is similar to naturally regenerated seedlings (Munson and Bernier 1993). However, these experiments were applied to evergreen conifer seedlings. Evergreen leaves act as both aphotosynthetic organ and a storage organ of nutrients. Is there any difference in root development as influenced by foliar habit, namely evergreens in spruce or deciduousness in larch?

Structure and function of root systems of larch and hybrid larch should have specific characteristics, even in very closely related species. Japanese larch has higher growth rate compared to the Dahurian larch or its hybrid larch (Koike *et al*. 2000), we hypothesize that the different fertilization regimes and dose rates may influence the specific root growth characteristics, biomass and nutrient uptake capacity of both the larch species. The objectives of this study were to assess root growth characteristics, biomass productivity and nutrient dynamics of larch species by different fertilization delivery schedule (exponential vs. conventional) and dose rates (Qu *et al*. 2003).

5.2 Materials and Methods

5.2.1 Plant material and cultural conditions

Seeds of Japanese larch and its hybridlarch were treated in 4°C for 10 days then germinated in agar media for two weeks. The seedlings were transplanted in plastic trays in the greenhouse attached to the Field Science Center of Hokkaido University, at ambient temperature, 18–25°C, humidity, 40–60% and photoperiod, 16hr. Photoperiod was adjusted by fluorescence lamps (FC4011 GL, Orderic CO., LTD, Tokyo, Japan) for plant culture of photosynthetic photo flux density (PPFD) of more than 300 μmol $m^{-2}s^{-1}$. Each tray held 32 rhizoboxes (Eiken Instruments CO., LTD, Tokyo, Japan) about 287cm^3 in cavity volume. Rhizoboxes were uniformly filled with an equal weight of clay-loam soil, peat moss and vermiculite (2:2:1 by volume). A single seedling was planted in one rhizoboxwrapped with aluminum foil. The trays were moved periodically in the growth cabinet to reduce edge effects.

5.2.2 Fertilization regimes

The four fertilizer regimes were tested by increasing amounts of nutrients applied conventionally or exponentially for 12 weeks during the growing season. Fertilization treatments consisted of one conventional treatment (i.e.10C) and three exponential treatments (i.e. 10E, 20E and 40E). The 10C was applied at a constant rate of (0.83 mg N $seedling^{-1}$ $week^{-1}$, 10mg N $seedling^{-1}$). 10E was equal to 10C in total amount (10mg N $seedling^{-1}$) but applied at an exponential rate of fertilization.20E (20mg N $seedling^{-1}$) and 40E (40 mg N $seedling^{-1}$) represented "middle" and "high" nutrient loading rates. Fertilizer treatments started one week after germination to avoid injuring young seedlings. A mixed nutrient solution (N: P: K (15:15:15) plus micronutrients) was added per week. This kind of fertilizer has been examined that acidification of soil occurred and no pH increased during short nursery seedling culture (Quoreshi *et al*. 1998, Qu *et al*. 2003). Although fertilizer doses varied with treatments, all seedlings received the same volume of solution (10 ml) per application. Calculation for weekly additions of exponential fertilization was based on an exponential function (Ingestad and Lund 1979):

$$N_T = N_S(e^{rt} - 1),$$

where N_T is the added amount of N during the whole growing season, N_S is the initial amount of N (0.2mg N, 0.26 mg N $seedling^{-1}$ for Japanese larch and its hybrid larch, respectively) in the seedling at the start of fertilization, r is the relative additional rate required to increase N_s, T is the number of fertilization periods for this experiment. After it was determined for the 12 fertilizer applications, the amount of N to be added (N_t) was calculated from the following equation:

$$N_t = N_S\ (e^{rt} - 1)\ - N_{t-1},$$

where N_{t-1} was the cumulative amount of fertilizer added up to and including the last fertilization. The weekly applications of N to one seedling are shown in Table 5.1.

5.2.3 Plant harvesting, measurements and statistical analyses

Ten seedlings of each treatment were randomly harvested at 30, 54, 78 and 102 days after germination. After harvesting, the roots of seedlings were washed free from growing media, then photographs were immediately taken, subsequently they were separated into root and shoot for morphological measurement and for chemical analysis, only the ones that were harvested last, shoots were divided into needles and stem. Roots were scanned for making a photocopy. Root lengths were measured by a digital curvi-meter (Koizumi Sokki Mfg. Co., Ltd, Tokyo, Japan). Root areas were analyzed with Area Meas (Hongu, Akinori MYKA. Lab.1.01 Ver, 1995).

These samples then were put into oven to dry at 60°C for 48 hours. Total N of seedling was determined by a N-C analyzer (NC-900, Shimadzu, Osaka, Japan). SAS mixed model analysis of four nutritional treatments of two larch species were conducted on growth and nutrient parameters of the seedlings using the mixed model procedure (SAS Institute, Inc., 1996). The significant levels between the treatments were separated by least square mean.

5.3 Results and discussions

5.3.1 Root growth responses

The root system of seedlings in term of biomass differed among various treatments in the Japanese larch

Table 5.1 Weekly application of N on larch seedlings under conventional (C) and exponential (E) regimes for 12 weeks. Treatment 10C represented conventional fertilization regime at total dose of 10mg per seedling during growing season. Treatments 10E, 20E and 40E represented exponential fertilization regimes at total dose of 10mg, 20mg and 40mg per seedlings during the growing season, respectively. JL represented Japanese larch and HL represented hybrid larch

	N applied (mg N seedling^{-1})							
	10C		10E		20E		40E	
Weeks	JL	HL	JL	HL	JL	HL	JL	HL
1	0.833	0.833	0.093	0.078	0.114	0.094	0.136	0.111
2	0.833	0.833	0.127	0.108	0.164	0.138	0.207	0.173
3	0.833	0.833	0.172	0.149	0.235	0.202	0.315	0.269
4	0.833	0.833	0.234	0.207	0.338	0.297	0.480	0.418
5	0.833	0.833	0.317	0.288	0.486	0.437	0.729	0.651
6	0.833	0.833	0.431	0.399	0.700	0.642	1.110	1.013
7	0.833	0.833	0.585	0.554	1.004	0.943	1.690	1.576
8	0.833	0.833	0.795	0.768	1.444	1.385	2.572	2.451
9	0.833	0.833	1.080	1.066	2.076	2.034	3.916	3.814
10	0.833	0.833	1.470	1.480	2.984	2.989	5.961	5.933
11	0.833	0.833	1.990	2.054	4.290	4.390	9.074	9.230
12	0.833	0.833	2.710	2.850	6.167	6.449	13.813	14.360
Total	10mg		10mg		20mg		40mg	

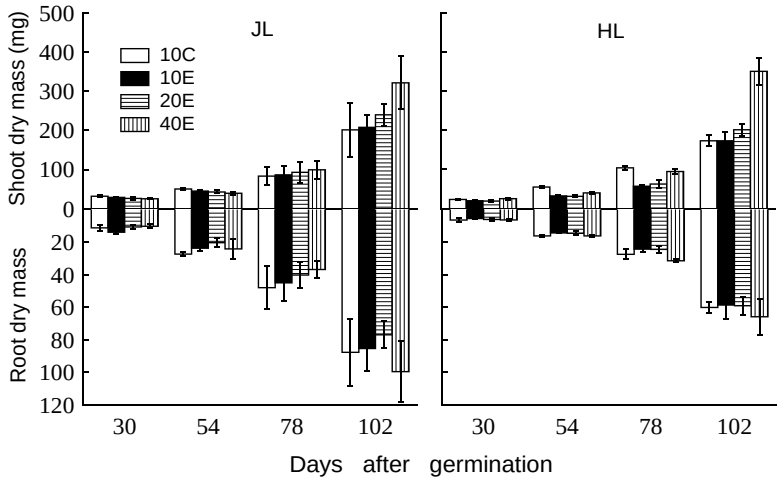

Figure 5.1 Dynamics of dry mass production of Japanese larch (JL) and hybrid larch (HL) seedlings raised for 102 days under various fertilization regimes. Vertical bars represent the standard error of its mean (SE).

and its hybrid larch. However, the growth pattern of two species showed a similar mode during the experiment (Figure 5.1). Among the four treatments, root dry mass in the Japanese larch and its hybrid larch showed significant differences ($P<0.05$) during the whole growing season. Conventional fertilizer treatment allocated more root biomass at the beginning of the growing season; in contrast, exponential fertilizer treatments allocated more root biomass at the end of the growing season. Root biomass of four treatments showed no significant differences in the Japanese larch or its hybrid larch by fertilization regimes.

The fertilization regimes induced different seasonal patterns of the growth and development of roots. Seedlings fertilized with a low dose conventionally (10C) and exponentially (10E) developed relatively longer roots (Figure 5.2) and had larger root surface areas (Figure 5.3) than that of those fertilized exponentially with a high dose (40E). The root length and root surface areas of 40E treated seedlings were smaller than that of those under other treatments, especially than that of the 10E treated seedlings. However, there were no significant differences in root surface areas among the four treatments in the course of growing season (Table 5.2).

During the whole growing season, root mass ratio (root mass as a fraction of total plant mass) of four treatments showed the same variant trend in both larch species (Figure 5.4). Root mass ratio for all treatments was increased from the beginning to the 54thday after germination, which indicated that the root growth is preferential during this period; root mass ratio declined from the 54thday to the late stage of the growing season, which indicated that nutrients mainly support shoot growth more than root growth. The major way in which plants can increase nutrient acquisition is by increasing

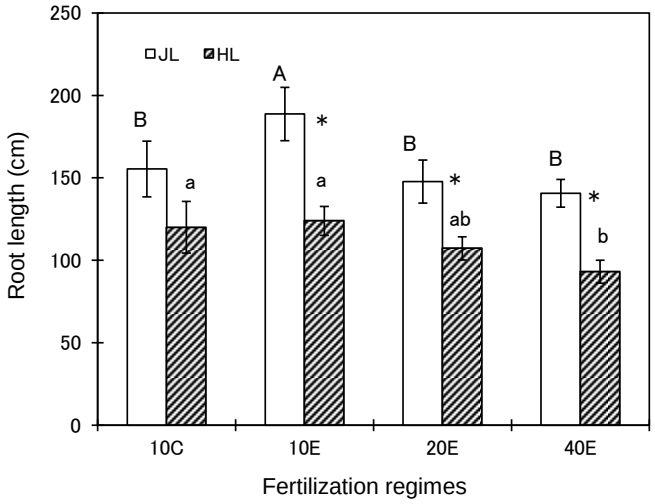

Figure 5.2
Root lengths of Japanese larch (JL) and hybrid larch (HL) seedlings under various fertilization regimes measured at the end of the growing season. Data within a series followed by the same upper case letter or lower case letter do not differ significantly ($P<0.05$) in the Japanese larch and its hybrid larch, respectively. A star symbolizes that there are significant difference ($P<0.05$) between two species under the same fertilization regime. Vertical bars represent the standard error of its mean (SE).

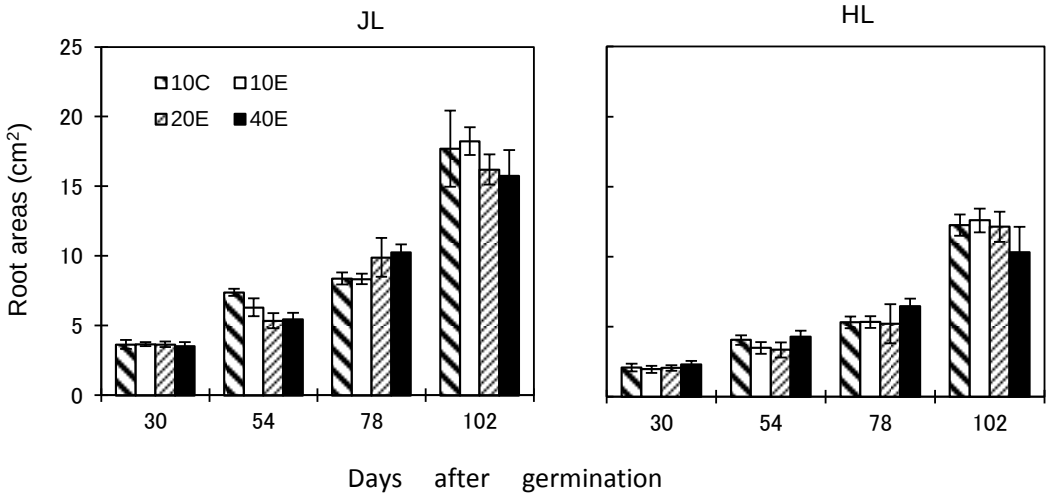

Figure 5.3 Root surface areas of Japanese larch (JL) and hybrid larch (HL) seedlings raised under various fertilization regimes during the growing season. Vertical bars represent the standard error of its mean (SE).

Table 5.2 Mean values of total root length, root surface areas, root biomass and shoot biomass for Japanese larch and its hybrid larch grown for 102 days at different nitrogen conditions. Values with the same letter (a, b) within row are not significantly different from each other at $P<0.05$ separated by Least Squares Means using SAS mixed model analysis. All values are means of six replicates

	10C	10E	20E	40E
Japanese larch				
Total root length (cm)	155.6*b*	188.7*a*	147.7*b*	140.7*b*
Root surface areas (cm^2)	17.7*a*	18.2*a*	16.2*a*	15.8*a*
Root biomass (mg)	87.6*a*	85.3*a*	86.2*a*	99.5*a*
Shoot biomass (mg)	201.3*b*	207.9*b*	239.8*b*	321.6*a*
Hybrid larch				
Total root length (cm)	120.0*a*	123.9*a*	107.3*ab*	93.0*b*
Root surface areas (cm^2)	12.3*a*	12.6*a*	12.1*a*	10.3*a*
Root biomass (mg)	60.1 *a*	58.0*a*	59.1*a*	65.8*a*
Shoot biomass (mg)	173.1*b*	173.1*b*	201.3*b*	350.7*a*

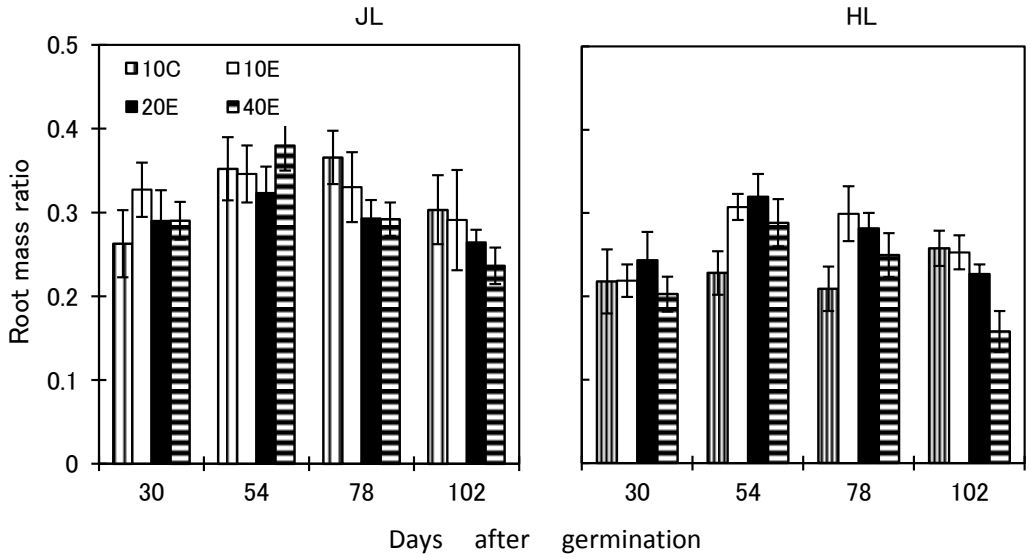

Figure 5.4 Root mass ratio of Japanese larch (JL) and hybrid larch (HL) seedlings raised for 102 days under various fertilization regimes and dosages. Vertical bars represent the standard error of its mean (SE).

the size of the root system. The relative size, expressed as the root mass ratio, is generally enhanced by growth at a low nitrogen supply (acclimation). Similarly, plants that have acclimated to a low nitrogen supply that typically have a high root mass ratio. The high potential in the relative growth rate that characterizes plants on fertile soil requires that a large fraction of the plant's resources be allocated to the leaves. This may be attributed to the treatment of 10C and 10E which induced larger root mass ratio in seedlings than that of 20E and 40E treated seedlings, therefore, the seedlings raised under 40E developed better aboveground than those of the other treatments. Because diffusion is the major process that delivers growth nutrients to plant roots, the main way of plant to augment nutrient is by increasing the size of root system (Lambers 1998). This may be attributed to the relatively lower nutrient supply associated with 10C and 10E, and that the major way in which they can increase nutrient acquisition was by increasing the size of the root system. These data indicated that different N levels (10mg, 20mg and 40mg) strongly affected root growth characteristics. At the same nutrient level (10mg), the delivery schedule had little effect on root biomass, root length and root surface areas of two larch species.

Though the root systems of 40E treated seedlings had less total root length and root surface areas by the end of growing season, they markedly developed much more short roots and mainly distributed the upper part of rhizoboxes, however, those of 10C and 10E developed relatively longer roots and were well-distributed throughout the rhizoboxes. In general, plants grown under high nutrient level tend to have a more dichotomous architecture (Taub and Goldberg 1996). Soil nutrient concentration affects both elongation and lateral initiation (Fitter 1991).

When grown under uniform conditions, root systems produce predictable and often recognizable architectures, which can be altered in equally predictable ways due to special changes in the environment. When roots develop in soil, the architecture alters, because their development is plastic (Fitter *et al*. 1998, Fransen *et al*. 1998). Plant adaptation to nutrients is the ability to position root growth in both time and space in such a way as to maximize the effectiveness of individual root elements for acquiring resources. Different doses of supplied nutrient induced significant differences in the distribution of the root system. Therefore, the pattern of the nutrient supply, i.e. conventional fertilization or exponential fertilization may have little effect on the root systems of the two larch species.

5.3.2 Responses on biomass

Different nutrient addition induced large differences in dry mass partition between the aboveground and belowground (Figure 5.1). Root dry mass between Japanese larch and its hybrid larch showed significant differences during the entire growing season. However, shoot biomass showed quite a different pattern between the two types of larch and the four treatments regarding roots. There was no significant difference in shoot biomass in the Japanese larch and its hybrid larchin any of the four treatments until the 78th day. Only at the final harvest, 40E treated seedlings had much more shoot mass (134% and 155%) as compared with seedlings grown under 20E and 10Eregimes in the Japanese larch. Shoot dry mass of 40E treated seedlings were significant different from other treated seedlings; shoot dry mass of 20E treated seedlings also were significant different from the 10C and 10E treated seedlings; however, there were no differences between the 10C and 10E.

Dry mass accumulation and rate of roots was higher than that of shoots at the beginning of growing season. After a 6-week fertilization, shoot dry massincreased

sharply. The increasing rate was higher than that of the root, 40E treated seedlings of the Japanese larch increased shoot dry mass about 8 times in a 12-week fertilization stage than in a 6-week fertilization, but the roots only increased 4 times in dry mass (Figure 5.1). It may be said that during this period nutrients are more supportive of the shoots than roots. According to the data of the root mass ratio, it may conclude that the larch seedlings utilize nutrients mainly to root growth at the beginning of growing season, after that, supported more nutrients for shoot growth. With the increase in shoot growth, photosynthesis capacity became higher and induced more photosynthates to be allocated to the root and improve root growth. However, root growth was preferential in contrast with shoot growth under our fertilization regimes. The rate of root growth was more closely related to the dry mass of roots than to the dry mass of shoots. Duration of root growth, however, was most closely related to the dry mass of the shoot system. This indicated a priority of sources allocation with a close dependence of root growth on the amount of reserves in the roots.

Throughout the growing season, there was no significant difference in root dry mass accumulation for all treatments, demonstrating that root dry mass accumulation was not proportional to the nutrient supply. Seedlings treated with 10C and 10E allocated relatively more biomass to their roots, which may have been due to a low nutrient addition. It is well documented that plants relatively allocate more biomass to roots and less to their leaves when nitrogen is restricted (Brouwer 1983). Seedling growth under 40E allocated relatively more biomass to shoots, which may have beendue to the high nutrient addition. In fertile conditions, plants with high growth rates allocated a large fraction of photosynthates to the leaves (Chapin *et al*. 1990). By the end of the growing season, conventional and exponential fertilization of low nutrient levels showed no significant difference in total dry mass production of the two larches' seedlings.

The Japanese larch has a higher growth rate compared to the hybrid larch in fertile soil (Koike *et al*. 2000). A fast-growing population allocated significantly more dry mass to new shoots than to the slow-growing population, whereas slow-growing populations allocated more biomass to old shoots and new roots than fast-growing populations (Hawkins *et al*. 1998). In the Japanese larch seedlings, total biomass allocation was larger than that of the hybrid larch, which may have been due to its relatively high growth rate.

5.3.3 Dynamics in nutrient uptake

Figure 5.5 shows the course of the N content in seedlings during the growing season. In general, the content of N in the Japanese larch was larger than that of its hybrid larch. Exponential fertilization increased in the concentration and content of N of whole plant. In contrast, the N concentration of whole plant declined for seedlings treated with the conventional fertilization method during the growing season (Figure 5.6). By the 102th day, the N content and N concentration of the whole plant were much greater in the 40E treated seedlings than in the 10C and 10E treated seedlings. Exponential fertilization (10E) increased the N uptake more than the equivalent amount of conventional fertilization (10C).

It demonstrated that the levels of fertilization regimes affected the N content of a two-larch seedling; conventional and exponential fertilization regimes resulted in different ways for N allocation. Exponential

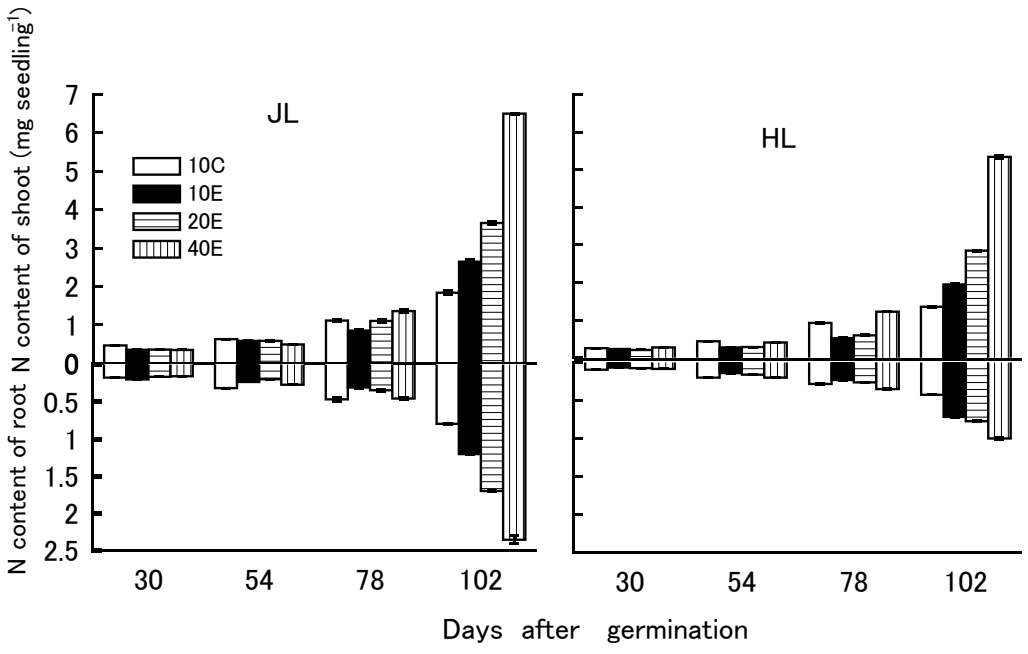

Figure 5.5 Seasonal dynamics of N content of Japanese larch (JL) and hybrid larch (HL) seedlings raised from seeds for 102 days under four fertilizer regimes. Vertical bars represent the standard error of its mean (SE).

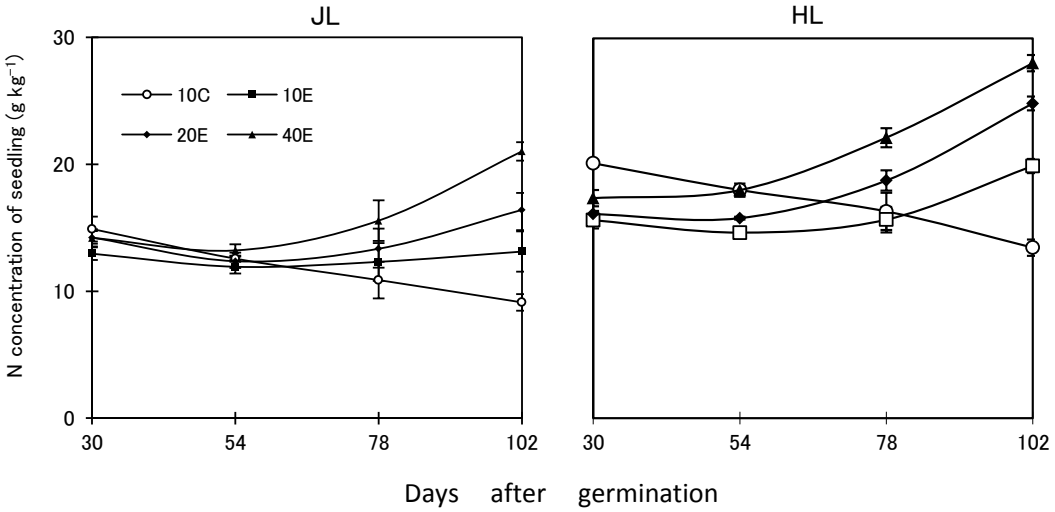

Figure 5.6 Seasonal changes of N concentration of Japanese larch (JL) and hybrid larch (HL) seedlings grown under various fertilization regimes and dosages. Vertical bars represent the standard error of its mean (SE).

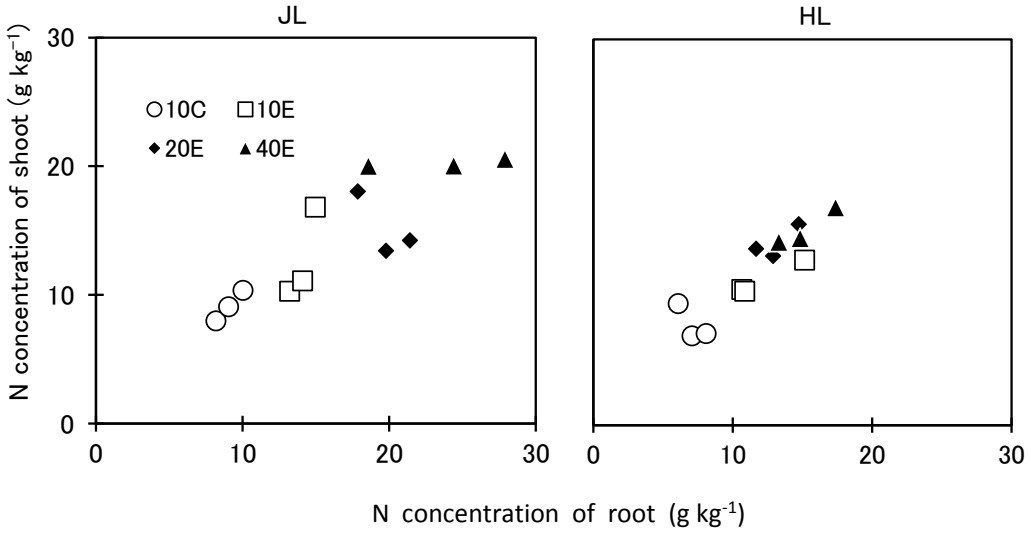

Figure 5.7 The relationship of N concentration between root and shoot of Japanese larch (JL) and hybrid larch (HL) seedlings under various fertilization conditions.

fertilizer simulated the natural supply and acquisition of nutrients for plants (Ingestad and Lund 1986). It matched the exponential growth of seedlings and in fact exceeded that of the conventional fertilizer. Under exponential fertilizer conditions, the N uptake of seedlings increased exponentially, while with conventional fertilizer, N uptake increased linearly.

Conventional fertilization is the delivered fertilizer that was given at constant dose levels throughout the growing season. It provided a relatively high nutrient supply in relation to the plant's demands at the beginning of the growing season, more so than that of the end of season. Consequently, with seasonal progress, nutrient dilution increased dry mass production, but declined the N concentration of seedlings grown under conventional fertilization treatment (Xu and Timmer 1998). This resulted in the N concentration of seedlings, when raised under conventional fertilization regime, declined in both larches (Figure 5.6).

The rate of nutrient uptake depended on both the concentration in the growing environment and the demand of the plant. The plant's demand is determined by its growth rate and the concentration of nitrogen in the tissue (Fitter *et al*. 1998). At a high internal nutrient concentration, nutrient uptake is down regulated. Despite this feedback mechanism, plants may show a type of luxury consumption of special nutrients (i.e., absorption at a higher rate than required to sustain growth), leading to the accumulation of nutrients. 40E treated seedlings increased root nutrient uptakes without increasing root dry mass, thus inducing the luxury consumption to build up internal nutrient reserves for the root.

Higher nutrient loading induced rich nutrient consumption, which may be characterized by increasing nutrient concentrations without significantly changing

the total dry mass. This higher nutrients reserve benefits root development and exploitation in the soil during critical establishment periods (Malik and Timmer 1995). The 40E treated seedlings did not significantly improve dry mass production of the root, but nutrient accumulation increased without a concomitant increase in root dry mass production. This may be a demonstration that 40E treated seedlings formed luxury nutrient consumption within the root.

The relationship of the N concentration between root and shoot is shown in Figure 5.7. The N concentration of root and shoot in 10C treated seedlings was lower than that those of 10E. It demonstrated that exponential nutrient supply was better matched with growth and nutrient uptake than that of conventional fertilization when promoting root growth and shoot growth of larch seedlings. By the end of the growing season, the 40E treated seedlings had developed a higher N concentration for both roots and shoots than the other treatments. However, interestingly, greater N was accumulated in roots compared to shoots of seedlings fertilized with 40E, suggesting that the roots may have stored N in other forms (e.g. amino acids or non-structural protein, amides), which may not have been available for transport to the shoots immediately (BassiriRad 2005).

The deciduous leaf habit of larch species suggests that as an adaptive strategy, this species can store nitrogen in the stem or root, which is an important resource for the next growing season. In the same fertilizer level (40mg), the N concentration of the root and shoot of the Japanese larch seedling was higher than that of its hybrid, which may indicate a higher growth rate of the Japanese Larch.

Different nutritional levels and fertilizer schedules significantly affected root growth and nutrient dynamics of two larch seedlings. It revealed that the larch species is sensitive to fertilization during the nursery culture. 10E treated seedlings increased nutrient uptake more than 10C treated seedlings. There were no significant differences in root dry mass, root length, root surface areas, root mass ratio between seedlings treated with 10C and 10E.However, they developed relatively longer roots and larger root surface areas than those treated with 40E. Seedlings grown under 40E regimes developed much more shoot dry mass, nutrient uptake than those of the 10E and 20E treated seedlings. Though the 40E treated seedlings developed a lot more fine roots (< 2 mm) it still was less than the 10E treated seedlings regarding root mass ratio, and root surface areas.

The low dose regimes (10C and 10 E) induced a relatively poor nutrient supply that may accelerate developing roots to excavate nutrients. The pattern of N content of both larch seedlings was closely related to the fertilization regimes. The N content of seedlings increased exponentially or linearly under exponential and conventional fertilizer schedules, respectively. The results demonstrated that the exponential fertilization technique matched more efficiently with the exponential growth and nutrient uptake of seedlings. In Japanese larch seedlings, biomass allocations, nutrient uptake and the size of root systems were all larger than in the seedlings of the hybrid larch. This may have been due to its relatively high growth rate.

Chapter 6
Ectomycorrhiza Symbiosis With the Root System
6.1 Introduction

In temperate and boreal forest ecosystems, most tree species form ectomycorrhizal association with diverse species of fungi in the Ascomycetes and Basidiomycetes. A number of studies have shown the importance of mycorrhizal fungi for the survival and growth of trees in forest ecosystem (Brundrett *et al*. 1996, Marx 1969, Browing and Whitney 1993). Mycorrhizae can enhance nutrient, water absorption by their mycelial networks, and protect the host plants against pathogens (Smith and Read 1997, Qu *et al*. 2010). Ectomycorrhizae can be found on about 90% of the trees in temperate and boreal forests (Le Tacon *et al*. 1992). In some forests under infertile condition, the growth of conifer seedlings areoften restricted by essential mineral nutrient in soil unless the mycorrhizae symbiosisis developed in their root system (Trofymow and van den Driessche 1991). Therefore, ectomycorrhizal association is necessary for successful establishment of newly planted seedlings in afforestation and reforestation sites. However, the influences of ectomycorrhizae on plant growth and nutrient uptake vary according to species of the ectomycorrhizal fungi (Jonsson *et al*. 2001).

Although some studies (Zhou *et al*. 1999, 2000, 2001a, 2001b, 2002, Yang *et al*. 1998) have shown that larch species are mostly associated with *Suillus* spp. and *Cenococcum geophilum* under natural conditions, little data is available describing the ectomycorrhizal development and growth of larch seedlings grown artificially and also inoculated with different ectomycorrhizal fungi. Yang *et al*. (1998) reported that Japanese larch may be associated with 3 or 4 types of mycorrhizal fungi depending on elevations. This plant is an obligatory ectomycorrhizal species, requiring mycorrhizal association for its survival and growth in the disturbed field.

Numerous *in vitro* systems of mycorrhizal synthesis have been developed and examined to measure the ability of fungi to form ectomycorrhizae (Fortin *et al*. 1983, Duddridge 1986a, b, Kottke *et al*. 1987, Wong and Fortin 1988, Wu *et al*. 1999, Vaario *et al*. 1999, Guerin-Laguette *et al*. 2000, Rincon 2001). In this study, we employed the modified Petri dish technique followed by the procedures described by Duddrige (1986a) and Qu. *et al*. (2003a). This method provides seedlings to form a natural shoot-root compartment by exposing the shoots to the atmosphere. Some studies (Straatsma *et al*. 1986) showed that the selective enclosure of the roots may be particularly important because certain stages of ectomycorrhizae development are influenced by gaseous factors. The rate and degree of colonization of the root surface is also influenced by the presence of carbohydrate in the synthesis medium. Based on the Duddridge's method (1986 a, b), we also examined the effects of small amount of external

carbon sources on the development of mycorrhizae.

The larch is an ectomycorrhizal species, requiring ectomycorrhizal association for its survival and growth in the disturbed field. Thus we are interested in whether ectomycorrhizal association is necessary for successful establishment of Japanese larch seedlings that are newly planted in afforestation and reforestation sites. There has been little previous research on how ectomycorrhizae affect the natural regeneration of Japanese larch. Some studies (Zhou *et al*. 2000, 2002, Yang *et al*. 1998) have shown that larch species are mostly associated with *Suillus* spp. in natural conditions, but little data is available describing the ectomycorrhizal development and growth of larch seedlings that are artificially inoculated with different ectomycorrhizal fungi.

Moreover, the hybrid larch is expected to allocate more photosynthate resources below ground than the Japanese larch, because the mother larch originates in the northeastern Eurasian Continent that has a short growth period. The hybrid larch may therefore store more carbohydrates in the root for survival, like alpine plants (Körner 1999). We have also examined the relation between the growth of hybrid larch seedlings and ectomycorrhiza formation, since there are natural regeneration and plantation practices in both larch species in northern Japan.

In this chapter, we include two experiments to discuss the ectomycorrhiza symbiosis with root system. For the first experiment, because of the low degree of host specificity of most ectomycorrhizal fungi, we introduced six different kinds of fungi to this trial. This study is an initial effort to improve our current knowledge of ectomycorrhizal fungi to associate with both larch species in order to develop further research.

In the second experiment, we investigated the effects of infection by ectomycorrhiza on the allocation pattern of ^{14}C-labelled photosynthates, in order to study the difference between the two species of larch. We used two kinds of inocula to study the growth of two larch seedlings in relation to early natural regeneration. One is the species *Suillus grevillei*, which is highly ecologically specific for *Larix* spp. (Duddridge 1986, Qu *et al*. 2003a); the other is forest soil inocula, which may include several kinds of ectomycorrhizal fungi.

6.2 *In vitro* Ectomycorrhiza formation

6.2.1 Materialsand Methods

6.2.1.1 Culture Media

During the ectomycorrhizal formation, sugar was added to the medium of Yang and Wilcox (1984); glucose (10 $g \cdot l^{-1}$) or was absent to the medium was discussed by Duddridge (1986a, b). Based on Duddridge's idea and our previous work (data unpublished), we added lower amount of glucose (2 $g \cdot l^{-1}$) in the media. In this petri dish method, we used vermiculite: peat substrate that allowed natural root development, formation of laterals. The substrate often used for seedling growth and artificial inoculation of containerized seedlings (Quoreshi and Timmer, 1998). Three different types of modified MMN solutions (Marx 1969) were used for the purpose of ectomycorrhizal fungal isolation, fungal inoculum culture and seedling culture. The original MMN media were slightly modified to fit our study objectives. The three modified solutions contained:

(a) $CaCl_2$,0.05g; NaCl, 0.025g; KH_2PO_4, 0.5g; $(NH_4)_2HPO_4$, 0.25g; $MgSO_4 \cdot 7H_2O$, 0.15 g; $FeCl_3$ (1%), 1.2ml; Thiamine HCl, 100 mg; Malt extract, 3g; Glucose, 10 g; stock solution of Micronutrient* (see below),1ml; deionized water 1000 ml; plus 15 g agar in case of agar media. The pH of the media was adjusted to 5.5 before autoclave.

(b) $CaCl_2$,0.05g; NaCl, 0.025g; KH_2PO_4, 0.25g; $(NH_4)_2HPO_4$, 0.125g; $MgSO_4 \cdot 7H_2O$, 0.15 g; $FeCl_3$ (1%), 1.2ml; Thiamine HCl, 100 mg; Glucose, 2 g; solution of Micronutrient* (see below),1ml; and deionized water 1000 ml. The pH of the media was adjusted to 5.5 before autoclave.

(c) $CaCl_2$,0.05g; NaCl, 0.025g; KH_2PO_4, 0.5g; $(NH_4)_2HPO_4$, 0.25g; $MgSO_4 \cdot 7H_2O$, 0.15 g; $FeCl_3$ (1%), 1.2ml; Thiamine HCl, 100 mg; Malt extract, 1.5g; Glucose, 4.5 g; stock solution of Micronutrient* (see below),1ml; streptomycin, 50 mg; chlortetracycline, 5 mg; deionized water 1000ml; and Agar, 15 g. The pH of the media was adjusted to 5.0 before autoclave.

* One liter micronutrient stock solutions contained: H_3BO_3 (2.86g), $MnCl_2$ (1.81g), $ZnSO_4$ (0.22g), $CuSO_4$ (0.08g) and Na_2MoO_4 (0.02g).

6.2.1.2 Plant materials

Before germination, the seeds of Japanese larch and its hybrid larch were soaked in distilled water at 4°C for 10 days. Then the seeds that were at surface sterilized by shaking them for 20 min in 30% H_2O_2 and were rinsed4-5 times with sterile deionized water. Germination was carried out aseptically on sterilized vermiculite-peat-sand media (vermiculite: peat: black sand =2:1:2). The seeds were incubated in a growth chamber maintainedat 20°Cunderphotosynthetic photon flux density of 150μmol $m^{-2}s^{-1}$ defuse fluorescent light (Toshiba, Tokyo) with a 16-h photoperiod until the seedlings are ready for inoculation.

6.2.1.3 Fungal materials

Six different species of ectomycorrhizal fungi were tested for synthesis of ectomycorrhizae with larch seedlings. The fungal isolates (*Russula emitica* (Schaeff.: Fr.) S. F. Gray (strain: 995), *Tricholoma saponaceum* (Fr.) Kummer (strain: 920), and *Lactarius hatsudake* (strain: 641)) were obtained from Biological Environment Institute, KANSO Co., Ltd. Japan. The isolates were collected from mixed pine forest in Honsu Island, Japan. The strains of *Suillus grevillei* (Klotz.) Sing. (strain: SG-1), and S*uillus laricinus* (Bk.) O.Kuntz (strain: SL-1) were stock culture from the Laboratory of Forest Resource Biology, Faculty of Agriculture, Hokkaido University. Both the species were collected from Mt. Komagatake and isolated from fruit body tissues. *Cenococcum geophilum* Fr. was isolated (medium c) from roots of *Picea glehnii* (Fr. Schm.) collected from a mixed larch and spruce stand

located at the experimental forests of Forestry and Forest Products Research Institute (FFPRI), Sapporo, Japan. The isolated fungal culture and the culture obtained from other laboratories were maintained on modified MNN agar medium (a) and periodically subculture for further inoculation studies.

6.2.1.4 Mycorrhizal synthesis

A potting substrate 2100 ml (containing vermiculite and peat moss (6:1), moisten with 1000 ml modified MMN nutrient solution (b) containing glucose (2%), were autoclaved at 121°C, 35 min. An electric cutter (Ultrasonic cutter SUW 30, Suzuki, co. Ltd., Osaka, Japan) was used to cut a slit into the side of petri dishes and their covers to make a space for seedling insertion. The petri dishes were aseptically filled with about 40ml of autoclaved substrate. Before inoculation, the six ectomycorrhizal isolates were cultured on agar plates containing MMN nutrient solution (a) for three to four weeks at 25°C in the dark. Three small plugs of (about 5-mm square each) actively growing mycelia from the margin of 3-week-old fungal cultures were transferred to petri dishes containing the substrate. The petri dishes were sealed with Parafilm and incubated about four weeks at 25°C in the dark.

During incubation, the fungal colonies were allowed to grow on the media and permeate maximum areas of the substrate (Figure 6.1 I). Six replicate plates of each fungal species were incubated for this experiment.

The germinated seedlings (2-week-old after germination) were surfacesterilized with H_2O_2 (1%, for 10min), rinsed three to four times with sterilized deionized water. This measure was taken to ensure that seedlings were free of contamination at inoculation. An aseptic individual seedling was transplanted per plate aseptically and the root system was carefully placed on surface of the growing colonies so that most laterals are in close contact with fungal mycelia (Figure 6.1 II). The seedling, including roots, was positioned horizontally. Three milliliters of MMN nutrient solution (b) was added to the substrate at this point. The shoot remains outside of the petri dish through the slit and the root remains in aseptic condition. The petri dish was sealed with parafilm and aluminum foil to keep the roots and fungus in darkness. The petri dishes were placed vertically in a plastic basket (Figure 6.2) and arranged in the growth cabinet (at 20°Cunder photosynthetic photon flux density of 150μmol $m^{-2}s^{-1}$defuse fluorescent light with a 16-h photoperiod) at the Experimental Nursery of Hokkaido University Forests. Cultures were monitored monthly and sterilized deionized water was added twice during culture period.

6.2.1.5 Harvest

Ten weeks after inoculation (Figure 6.1 III, IV), estimation of ectomycorrhizae formation and seedling growth were measured. Root systems of seedlings were photographed for the evidence of ectomycorrhizal formation and the seedlings were removed from culture plates. The seedlings were separated into root and shoot components and the roots carefully washed with water free of substrate. Ectomycorrhizal roots were excised and kept aside for further anatomical studies. The seedling shoots were oven dried (60°C, 48hr).

6.2.1.6 Evaluation of ectomycorrhiza

All root segments selected were soaked in distilled water and observed under a stereomicroscope at 20-80x magnifications for ectomycorrhizal evaluation. Ectomycorrhizal tips were confirmed by the color, form and size of ectomycorrhizae, the presence emanating hyphae and weft of mycelia on the root surface, the presence of rhizomorphs, the absence of root hairs using a stereomicroscope and a compound light microscope.

Preparation of roots for microscopic study was similar to the procedure described by Burn *et al*. (1995), Peterson (1991), and Murakami *et al*. (1999). Root segments of each type were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for overnight at room temperature and washed three times for 15 min each in the same buffer. After washing, the root samples were dehydrated in a graded ethanol series (25%, 50% 75%, 90% and 100%). They were then gradually embedded with epoxy resin at room temperature, then embedded in gelatine capsules and then the resin was polymerized. The sections (1μm thick) were cut with a glass knife on an ultramicrotome (Ultracut J; Austria). The sections were stained with a 1% solution of safranin. They were observed with a compound light microscope (BHS-2; Olympus, Japan).

Fresh mycorrhizas were also used for microscopic identification as described by Ursic and Peterson (1997). A fresh root segment was placed on a glass slide, and hand sections were made using sharp steel blade, stained with 1% (w/v) of aqueous toluidine blue and with 0.1% (w/v) of cotton blue in 10% (v/v) lactophenol/water and observed. At least 10 tips of each type were sectioned for anatomical study. Transverse sections and vertical sections were observed to verify the presence of mantle hyphae and Hartig net.

6.2.2 Results and discussion

All fungal species tested apparently formed a typical ectomycorrhizae symbiosis with both Japanese larch and its F1 larch under the present test conditions (Figure 6.3). Mycelial spread and ectomycorrhizal formation with well developed rhizomorphs and emanating hyphae were shown in Figure 6.1 and 6.3.The short ectomycorrhizal roots of Japanese larch with *S. grevillei* were completely enveloped with cottony hyphate growing on the mantle (Figure 6.3 a, c). Among the two *Suillus* spp., *S. grevillei* had faster growth (infection rate 100% of total root tips) and spread better on the artificial media than *S.laricinus* (infection rate 92% of total root tips). The relatively high infection rates of two *suillus* species demonstrated that they were suitable species for developing ectomycorrhizal association with Japanese larch and its hybrid larch. This may be due to the reason that they were isolated from the larch forest and had a very close relationship with the larch. A thick black and dark brown straight emanating hyphae are the characteristics of *C.geophilum* (Agerer 1994). *C. geophilum* only

colonized near the inoculum plugs (Figure 6.3 f, g), which may due to *C.geophilum* being a slow growing fungus. Microscopic examinations of the root system of all seedlings showed that the most laterals that are in contact with growing colony are completely enveloped by the fungal hyphae (Figure 6.3).

Mantle colors of the each mycorrhizae were similar to colony in pure culture in most of the cases. Infection status observed after morphological and anatomical observation showed the evidences of successful ectomycorrhizal formation in all species (Figsure6.3 and 6.4).Apparently, the best infection occurred with *S. grevillei* (Figure 6.3 a-e) with two plant species followed by *S. laricinus* (Figure 6.3 i) and *T. saponaceum* (infection rate 77% of total root tips) (Figure 6.3 j). Other species of isolates, such as *L. hatsudake* (Figure 6.3 m), *R. emitica* (Figure 6.3 k), and *C. geophilum* (Figure 6.3 f, g) showed relatively slower growth in this culture condition. The infection rate of *L.hatsudake*, *R. emitica*, and *C. geophilum* were 63%, 48% and 41% of total root tips, respectively. However, successful mycorrhizae formation was observed with both plant species with these three species. *L. hatsudake* produced smooth ectomycorrhiza (Figure 6.3 m).

Several attempts of anatomical studies provided the evidence of ectomycorrhizae formation with *L.hatsudake* (Figure 6.4 A, G, H). Microscopic examination revealed the presence of mantle some times in two layers, an inner layer consisting well stained and compact hyphae, and an outer region with loose and lightly stained external mycelia (Figure 6.4). These preliminary results indicate the six fungi were able to colonize the roots of Japanese larch and hybrid larch F_1at the end of this experiment, once hyphae had contacted the root surface.

Figure 6.5 shows the shoot biomass accumulation of both larch seedlings at the end of culture period. Growth in all seedlings was not satisfactory as we expected after 10 weeks. This was probably due to cultivation system and N limitation. However, the shoot growth showed some variations depending on the fungal species inoculated. The reduced biomass growth compared to the expected 10-week growth for larch probably attributed to exhaustion of nutrients in this culture system since both plant and fungal growth required substantial amount of N. Nevertheless, we do not have a clear explanation why these differences in inoculation affected seedling growth, but it partly may be due to the small amount of samples and lack of non-inoculated control seedlings. Unfortunately, we could not provide the root biomass because of loss in samples during mycorrhizal evaluations.

This study demonstrated that *S. grevillei*, *S. laricinus*, *R. emitica*, *T. saponaceum*, *C. geophilum* and *L. hatsudake* can develop mycorrhizal association on roots of Japanese larch and F1 hybrid larch under present artificial culture conditions. The fungal species varied in both spreading on vermiculite-peat media and the ability to form ectomycorrhizae. We could get the initial step for studying nutritional association in larch-fungus based on Duddridge's method.

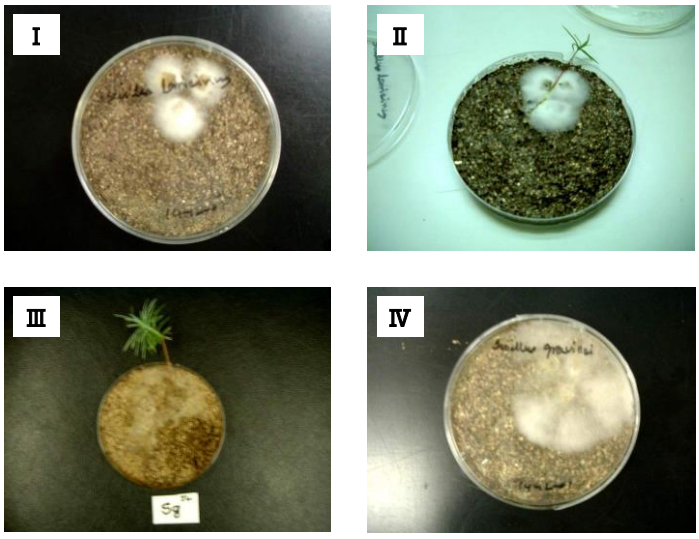

Figure 6.1
Macroscopic view of fungal inoculum growth on vermiculite: peat media in petri dishes and inoculated larch seedlings. I and II *Suillus laricinus* grew on vermiculite: peat medium one month and transferred seedlings to petri dish. III and IV Harvest seedlings growing with *Suillus grevillei* after ten weeks inoculation.

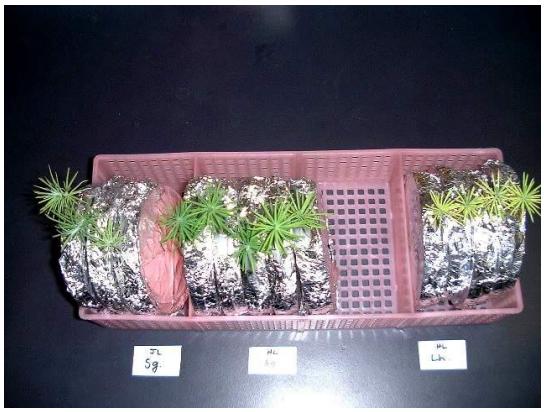

Figure 6.2
Aseptic synthesis of ectomycorrhizae. Tree seedlings growing in petri dishes placed in plastic box inside a growth cabinet.

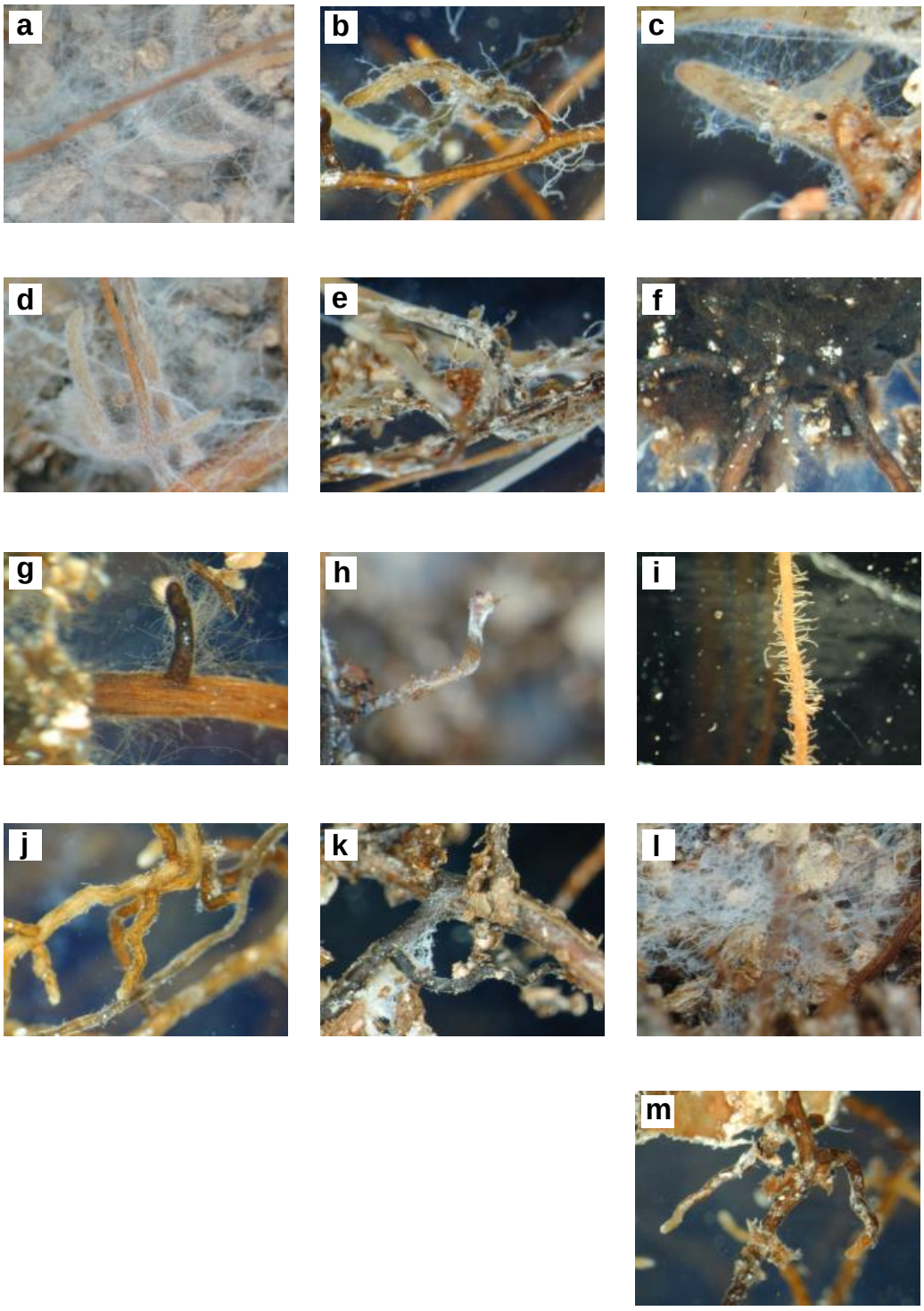

Figure 6.3

Stereomicroscpoic view showing ectomycorrhize formation between the roots of two larch species and six different ectomycorrhizal fungal isolates. Figs. a-c. Ectomycorrhizal roots of Japanese larch with *Suillus grevillei*. Short roots are completely enveloped with cottony hyphae growing on the mantle (a, c) and with distinct rhizomorph (c). Figs. d-e. *Suillus grevillei* ectomycorrhizae on F1 larch roots with fungal hyphae and rhizomorphs. Figs. f-g. Compact *Cenococcum geophilum* fungal colony growing on the substrate (f) and formed ectomycorrhizae showing characteristic black emanating hyphae (g). Fig. h. A single root segment colonized by *Tricholoma saponaceum* on Japanese larch. Fig. i. A non-mycorrhizal root segment F1 larch with abundant root hairs. Figs. j-m. Ectomycorrhizal roots with attached fungal hyphae between *Tricholoma saponaceum* and F1 larch (j), *Russula emitica* and Japanese larch (k), Suillus laricinus and F1 larch (l), and *Lactarius hatsudake* and Japanese larch (m).

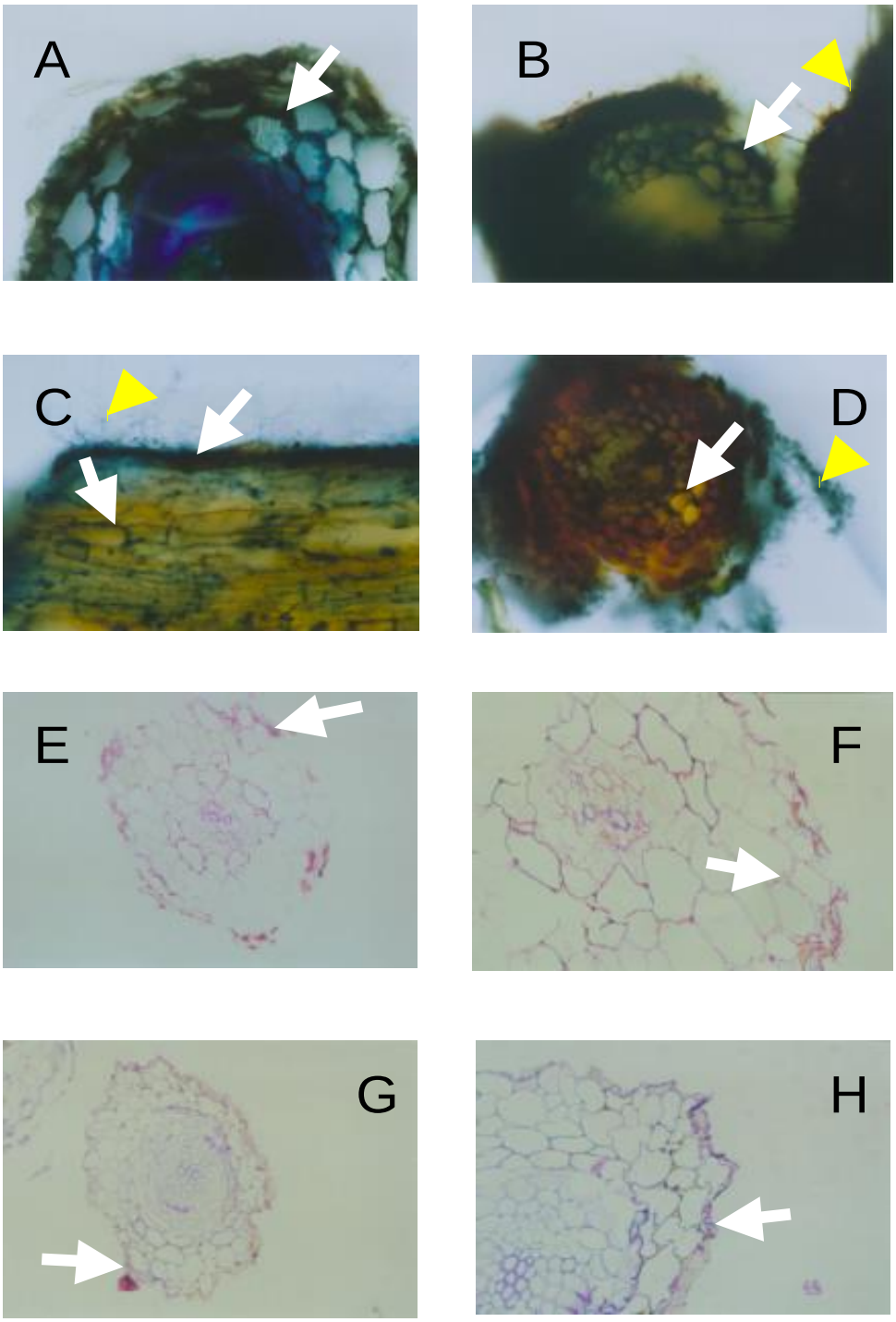

Figure 6.4

Light microscopic view showing anatomical features of ectomycorrhizae formation. Fig. A. Transverse section (100x) of short roots from inoculated *Lactarius hatsudake* and Japanese larch, showing the dark color outer mantle and the arrow indicates Hartig net hyphae. Fig. B. Showing a thick mantle (100x) of *Cenococcum geophilum* with distinct Hartig net (arrow) and partially separated mantle (yellow arrowhead) with septate mycelia. Fig. C. Longitudinal section of roots from *Suillus grevillei* and Japanese larch mycorrhizae (100x) showing hyphal growth from the mantle (yellow arrowhead), a well developed mantle surface, and Hartig net formation. Fig. D. *Tricholoma saponaceum* – F1 larch, showing a broken part of mantle surface (yellow arrowhead) and formation of Hartig net (arrow). Figs.E-H. Transverse section taken by using glasscutter from roots of *Suillus grevillei* - Japanese larch (E, F) and *Lactarius hatsudake* F1 larch (G, H) ectomycorrhizae, showing the penetration by the Hartig net and thin mantle surface (arrows).

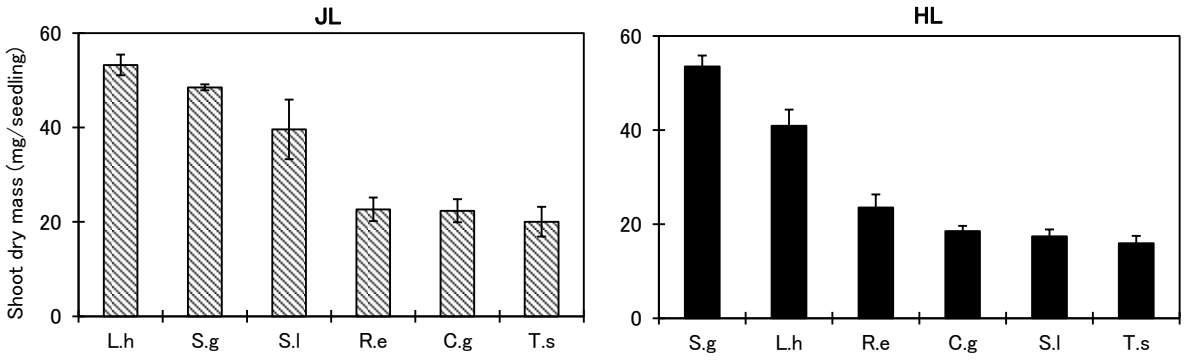

Figure 6.5 Shoot biomass of Japanese Larch (JL) and its hybrid larch (HL) seedlings inoculated with six different ectomycorrhizal fungi. The abbreviation Sg, Lh, Re, Cg, LI, and TS stands for *Suillus grevillei*, *Lactarius hatsudake*, *Russula emitica*, *Cenococcum geophilum*, *Suillus laricinus*, and *Tricholoma saponaceum*, respectively.

6.3 Allocation of carbon in ectomycorrhizal larch seedling

6.3.1 Materials and Methods

6.3.1.1 Plants and fungal provenances

Seeds of Japanese larch and its hybrid larch were supplied from the Uryu Experimental Forest of Hokkaido University. The ectomycorrhiza-forming fungus, *S. grevillei* was collected from Mt. Komagatake (42º04'N, 140º42'E, 1133m in altitude, surface soil pH was ca. 5.6) and was isolated from fruit body tissues. Fresh soil from the experimental forests and inucula medium soil was sieved, and its pH was measured by a pH meter (MP 220, TOA Electric Co., Ltd. Tokyo). The strains of *S. grevillei* (Klotz.) Sing. (strain: SG-1) was stock culture of Laboratory of Forest Resource Biology, Hokkaido University. The forest soil was collected from a ca. 50-year Japanese larch plantation in the Tomakomai National Forest of Hokkaido University.

6.3.1.2 Inocula preparation and plant inoculation

The standard growing soil was a mixture of loam, clay-loam and peat moss (volume ratio 2:2:1) and was autoclaved at 121°C for 30 min. The pH of this soil was ca. 6.2 at the start of the experiments. The forest soil (soil surface pH was 5.7) was passed through a 2 mm sieve; it included very small pieces of ectomycorrhizal roots. The forest soil medium (hereinafter, FM) were mixed with seven times as much standard soil and forest soil. *S. grevillei* inoculums was grown in liquid MMN medium for one month and mixed with vermiculate. *S. grevillei* inoculums medium (hereinafter, SM) and no ectomycorrhiza medium (hereinafter, NM) were mixed with vermiculate (including and without *S. grevillei*) and standard soil in the same ratio as in the FM treatment.

Seeds of Japanese larch and its hybrid larch were kept at 4°C for 21 days and then germinated in the medium comprising vermiculate, peat moss and clay-loam soil (3:1:2 by volume) for six weeks. The seedlings were transplanted into plastic pots (diameter 10.5cm, cavity volume 500 ml) in a greenhouse attached to the Field Science Center of Hokkaido University, at ambient temperature (18–25°C), humidity, 40–60% and 16hr photoperiod. The photoperiod was maintained by fluorescence lamps (FC-4011 GL, Orderic CO., LTD, Tokyo, Japan) to provide for the plants a photosynthetic photon flux density (PFD) of 300 – 400 μmol $m^{-2}s^{-1}$.

6.3.1.3 ^{14}C pulse-tracking

After the seedlings had been cultured for 110 days, shoots were enclosed individually in small, gas-tight, clear plastic bags and exposed to a pulse of $^{14}CO_2$ for one hour under natural light conditions. The $^{14}CO_2$ was liberated by mixing 1ml of 0.18mM $NaHCO_3$ with 370 KBq $NaH^{14}CO_3$ and 1ml of 300mg L^{-1} $HClO_4$. The seedlings were harvested at 0, 6 and 24 hr after exposure. Total ^{14}C was measured as described by Shinano *et al*. (1996) and Qu *et al*. (2004b).

6.3.1.4 Harvest and data analysis

The pots were moved in the greenhouse to reduce edge effects each week. CO_2 exchange was measured with a portable open-system IRGA (LI-6400, Li-Cor, Lincoln, NE, U.S.A.). Three seedlings from each treatment were sampled 110 days after inoculation. Light at saturating intensity (1000 μmol $m^{-2}s^{-1}$) was provided by an LED light source (Li-Cor 6400-02B) with red/blue light and the CO_2 concentration was controlled by the Li-Cor LI-6400 CO_2 injection system. Measurements were made at ambient values of air temperature and relative humidity. The initial slope of the A-Ci curve shows carboxylation efficiency (CE) i.e. the activity in Rubisco (Farquhar and Sharkey, 1982). Moreover, the CO_2 saturated photosynthetic rate corresponds to the rate of RuBP regeneration, which is directly connected with the translocation rate of photosynthates in chloroplast via Pi (Sharkey 1985).

Stomatal limitation was estimated as follows (Sharkey 1985):

$$Ls\ (\%) = (1 - Ai/Aa) \times 100,$$

where Ai is the net photosynthetic rate (Pn) at Ci and Aa is the Pn at ambient CO_2 level with no stomatal limitation. The needle area was determined

immediately after measurement of gas exchange. All harvested seedlings were separated into needles, stems and roots. These components were dried to constant mass at 80°C for 48 hrs, then milled for homogeneity. The P content was determined after acid digestion by H_2SO_4 and H_2O_2 according to the method described by Murphy and Riley (1962).

6.3.1.5 Evaluation of ectomycorrhiza

All root segments selected were soaked in distilled water and ectomycorrhizal characteristics were observed under a stereomicroscope at 20-80xmagnifications and a compound light microscope. Ectomycorrhizal tips were confirmed by the color, form and size of ectomycorrhizae, by the presence of rhizomorphs and absence of root hairs. These roots were prepared for microscopic observation according to the procedure of Burn *et al*. (1995) and Peterson *et al*. (1991). Anatomical analyses was followed the procedure of Qu *et al*. (2003a).

6.3.1.6 Statistics

Treatment effects on biomass, including seedling, needle, stem and root biomass, root/shoot ratio, ^{14}C allocation in seedling, needle, stem and root, photosynthetic rate, carboxylation efficiency, stomatal limitation were detected by separate one-factor analysis of general liner model of SAS (SAS Institute, Inc., 1996) at the final harvest. The significant levels between the treatments were separated by least square mean.

6.3.2 Results

6.3.2.1 Colonization

Colonization of root systems was evaluated by removing seedlings from their pots after 110 days. Mycelia were clearly visible in roots inoculated with forest soil and *S. grevillei*. FM seedlings produced the greatest number of infection points in both the Japanese larch (Figure 6.6) and hybrid larch. At least two morph types of ectomycorrhiza were found in FM roots by stereomicroscope (Figure 6.6). The photo of anatomical analyses was showed for further evidence of ectomycorrhiza formation in the larch roots. The infection rates of FM seedlings were nearly 100% for the Japanese larch and hybrid larch; for SMseedlings the rates were 51.2% for the Japanese larch and 36.7% for the hybrid larch. Short roots were completely enveloped with cottony hyphae growing on the mantel (Figure 6.7). No ectomycorrhiza were found in roots of NM seedlings for the two species.

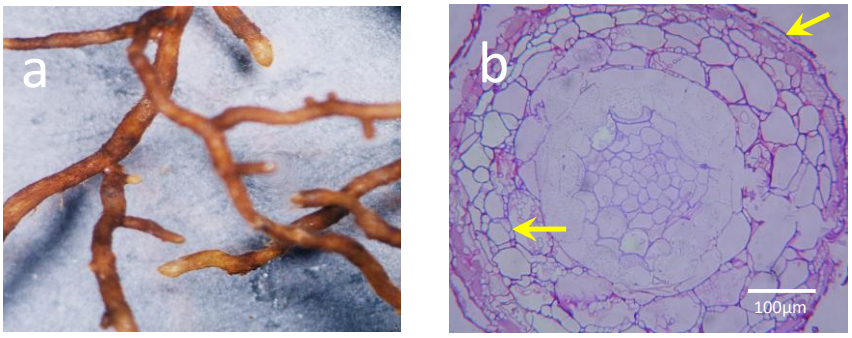

Figure 6.6 Stereomicroscopic view (a) and light microscopic view (b) showing ectomycorrhizae formation between roots of the Japanese larch seedlings colonized by larch-forest soil inoculums. (a) Observation of ectomycorrhizal roots by stereomicroscope. (b) Transverse section taken by using glasscutter from roots of FM seedlings, which showing the penetration by the Hartig net and thin mantle surface (arrows). FM represents seedlings colonized by larch-forest soil inoculums.

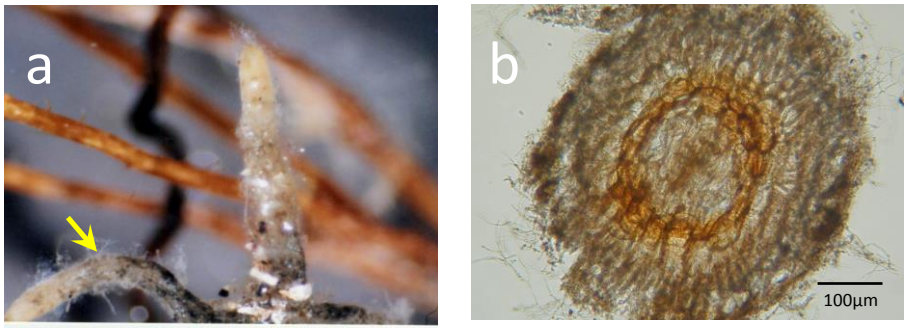

Figure 6.7 Stereomicroscopic view (a) and light microscopic view (b) showing the ectomycorrhiza formation between the roots of the Japanese larch and *Suillus grevillei* after 110-day inoculation. (a) Observation of ectomycorrhizal roots by stereomicroscope. Short roots are completely enveloped with cottony hyphae growing on the mantel. (b) Transverse section taken by using glasscutter from roots of SM seedlings, which showing the penetration by the mantle surface (arrows). SM represents seedlings colonized by *S. grevillea.*

6.3.2.2 Plant growth response to inoculation

The effect of infection of mycorrhizae on plant growth was determined by comparing plant dry mass at the end of the experiment. For both the Japanese larch and its hybrid larch, needles, stems and roots of FM seedlings were significantly larger than in SM or NM seedlings after 110 days (Figure 6.8). However, *S. grevillei* did not significantly affect the shoot growth or root growth of seedlings of either larch species. FM seedlings were taller and had larger root systems, and both shoots and roots were of greater dry mass. But the ratios of root to shoot mass were smaller in FM seedlings for both two-larch species. Overall, the Japanese larch seedlings had significant greater dry mass than the hybrid larch.

The relation between the net photosynthesis rate (Pn =A) and the intercellular CO_2 concentration (Ci) is shown in Figure 6.9. Based on the A-Ci curve, the lower carboxylation efficiency (CE) of NM showed lower activity in Rubisco at ambient CO_2 conditions. For both larch species, the maximum photosynthesis rate (Pmax) was significantly higher ($P<0.05$) in FM and SM (=EM; ectomycorrhizal Medium) seedlings than in NM seedlings. RuBP regeneration for SM and NM seedlings were suppressed at high CO_2 concentration compared to FM seedlings. Stomatal limitation of photosynthesis was significantly greater ($P<0.05$) in NM seedlings than in EM seedlings of the Japanese larch and hybrid larch (Table 6.1).

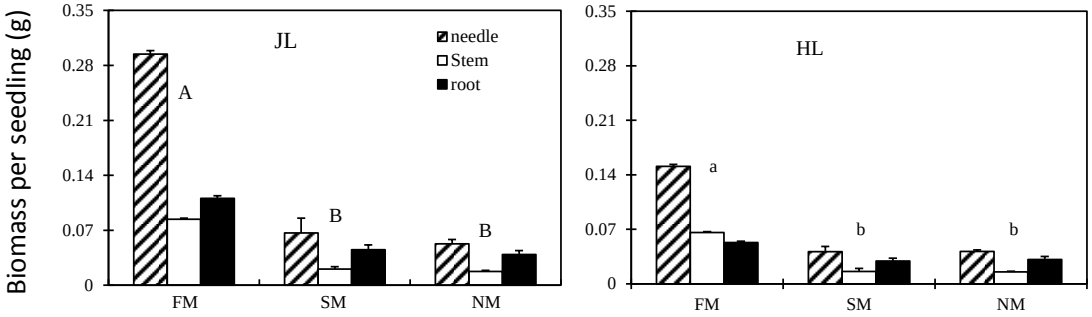

Figure 6.8 Needle, stem and root biomass for the Japanese larch (JL) and its hybrid larch (HL) under ectomycorrhizal and non-ectomycorrhizal treatments and grown for 110 days. FM, SM and NM respectively represent seedlings colonized by larch-forest soil inoculums, *Suillus grevillei*, or no fungi. Data within a series followed by the same letter do not differ significantly ($P<0.05$). Vertical bars represent the standard error of a mean (+SE).

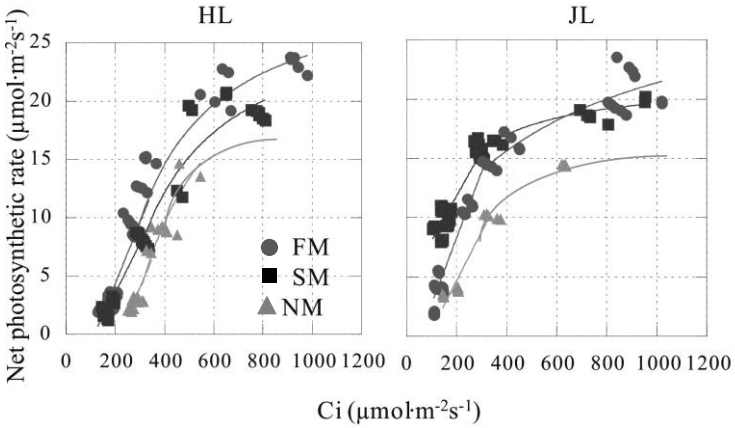

Figure 6.9 Relations between photosynthesis and the intercellular CO_2 concentration in two larch needles of FM, SM and NM seedlings. FM, SM and NM respectively represent seedlings colonized by larch-forest soil inoculums, *Suillus grevillei*, or no fungi.

Table 6.1 The mean value of carboxylation efficiency (CE), max photosynthesis rate (Pmax) and stomatal limitation (Ls) for Japanese larch (JL) and hybrid larch (HL) under FM, SM and NM treatments. FM, SM and NM represented the seedlings were colonized by larch-forest soil inoculums, *Suillus grevillei*, or non-colonized, respectively. Data within a series followed by the same letter do not differ significantly ($P<0.05$). Standard error was followed its mean

	JL			HL		
	CE	Pmax	Ls(%)	CE	Pmax	Ls(%)
FM	0.046(0.004)	23.6(1.3)a	49.0(8.6)b	0.050(0.008)A	22.4(2.3)a	46.2(3.0)b
SM	0.055(0.012)	23.7(0.4)a	58.3(3.0)b	0.041(0.002)A	22.7(0.9)a	50.1(8.4)ab
NM	0.038(0.021)	14.4(1.2)b	61.0(4.8)a	0.03(0.006)B	14.2(0.6)b	59.6(0.4)a

6.3.2.3 Carbon allocation

The fraction of carbon allocated to roots was calculated as the sum of ^{14}C in the root tissue divided by the total ^{14}C recovered in root, stem and needle fractions. Differences in this fraction between FM-NM and SM-NM were used as estimates of the carbon demand by the fungus. The total amount of ^{14}C incorporated in seedlings was significantly higher in FM seedlings than in NM seedlings of both the Japanese larch and hybrid larch at times 0hr, 6hrs and 24hrs after exposure to the radioisotope for one hour (Figure 6.11). SM seedlings incorporated more ^{14}C than NM seedlings, but the difference was not statistically significant. Japanese larch seedlings incorporated more ^{14}C than hybrid larch seedlings in all the treatments.

At the first harvest (0hr), there was significantly more ^{14}C in the root in FM seedlings than in SM and NM seedlings of Japanese larch; however, there had no such differences in the hybrid larch. The disintegration per min (hereinafter, DPM) per mg (dry mass) of ^{14}C had no differences neither in needle, stem nor root in Japanese larch seedlings and hybrid larch root (data not shown). At the second harvest (6hr), FM and SM seedlings had incorporated more ^{14}C in stems and roots than in NM seedlings for Japanese larch; the DPM per mg of seedlings was also higher in FM and SM specimens than in NM Japanese larch seedlings. At the final harvest, SM seedling of the Japanese larch and its hybrid larch respectively allocated 2.6% and 2.5% more ^{14}C to root than did the NM seedlings. In contrast, FM seedlings allocated 6. 5% more (Japanese larch) and 18.0% more (hybrid larch) to the stem than did the NM seedlings (Figure 6.11). FM and SM seedlings allocated more ^{14}C than NM seedlings after 24 hr. of chasing.

Immediately after exposure (0 hr), ^{14}C mainly located in the needles and allocation to root and stem was very low. The proportion of photosynthates allocated to the root increased sharply after 24hrs in NM and SM seedlings. However, distribution to the root after 24hrs was lower in FM than in NM or SM for either larch (Figure 6.11). Although a relatively lower proportion was distributed to the root in FM seedlings after 24h, the proportion in the stem was significantly higher (Figure 6.10, $P<0.05$).

6.3.2.4 Nutrient uptake

FM and SM seedlings showed higher assimilated nitrogen (N; mg) than NM seedlings. The amount of N in the Japanese larch was about 3.8 times higher in needles and 4.3 times higher in roots of FM seedlings than NM seedlings; for SM seedlings these ratios were 1.5 and 1.8 relative to NM seedlings (data not shown).

The effect of fungi treatment on the total phosphorus concentrations (P) in plants generally paralleled to the effects on dry mass. The P content of plant shows significant difference in the three treatments for both larch species (Figure 6.11, $P<0.05$). Growth promotion and increased P were largest in the forest soil inoculum treatment in both the Japanese larch and hybrid larch. FM seedlings contained two or three times as much P as NM seedlings after 110 days. The P in the root was about 1.7 times that in the needle for both larches during the growing season.

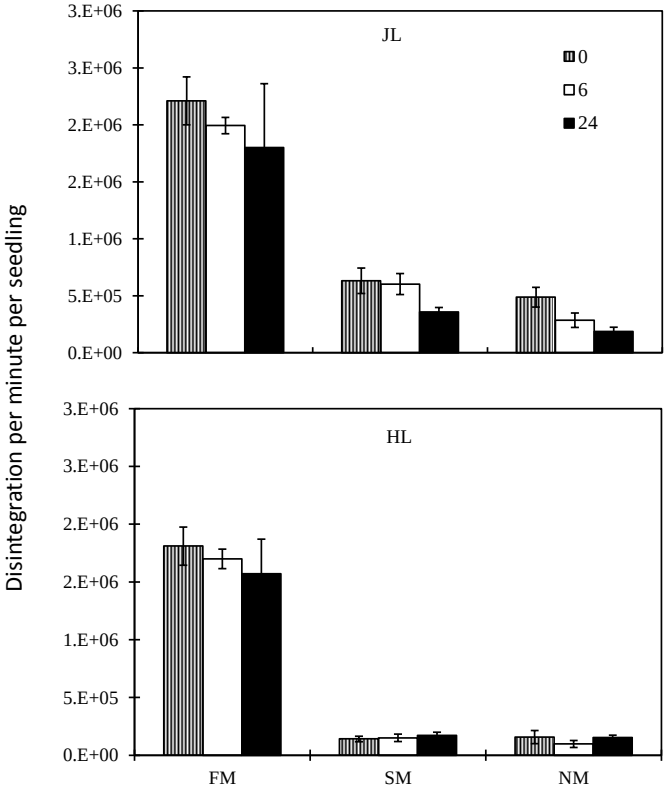

Figure 6.10 Disintegration per minute (DPM) of ^{14}C from whole seedling of the Japanese larch (JL) and hybrid larch (HL) after 0, 6 and 24h after exposure of ^{14}C (1h). FM, SM and NM respectively represent seedlings colonized by larch-forest soil inoculums, Suillus grevillei, or no fungi. Vertical bars represent the standard error of a mean (+SE).

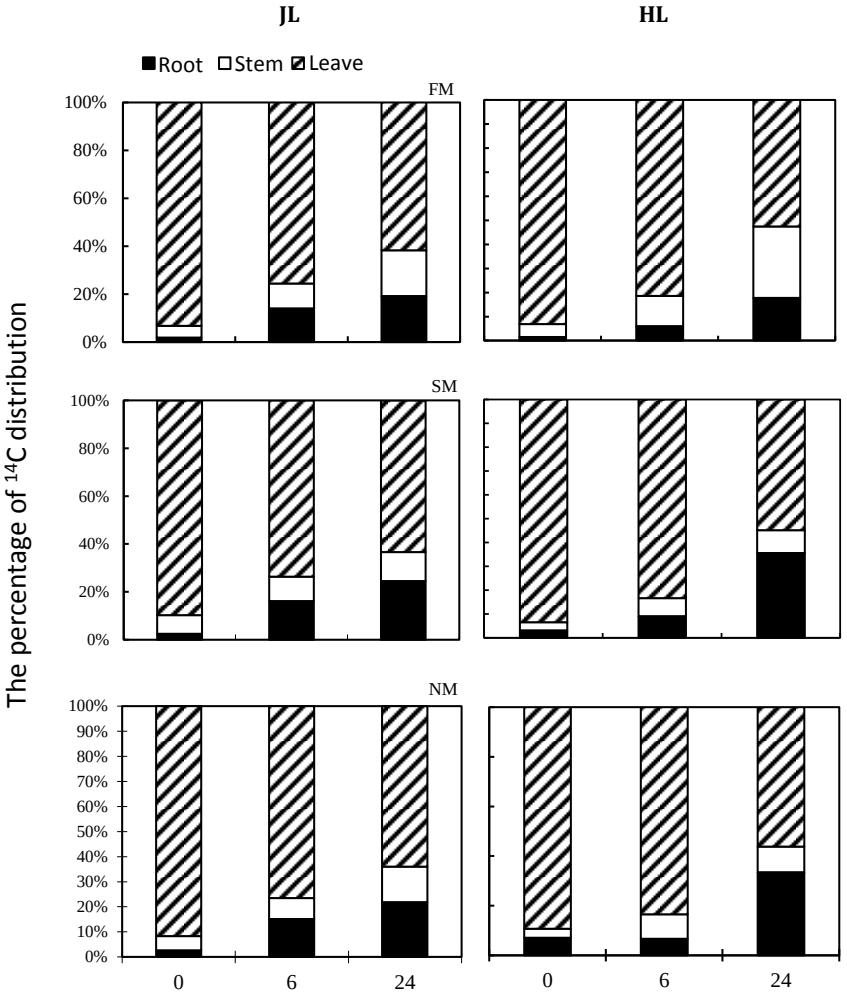

Figure 6.11 The percentage of ^{14}C distribution ratio in needle, stem and root of the Japanese larch (JL) and hybrid larch (HL) after 0, 6 and 24h after exposure of ^{14}C(1h). FM, SM and NM respectively represent seedlings colonized by larch-forest soil inoculums, *Suillus grevillei*, or no fungi.

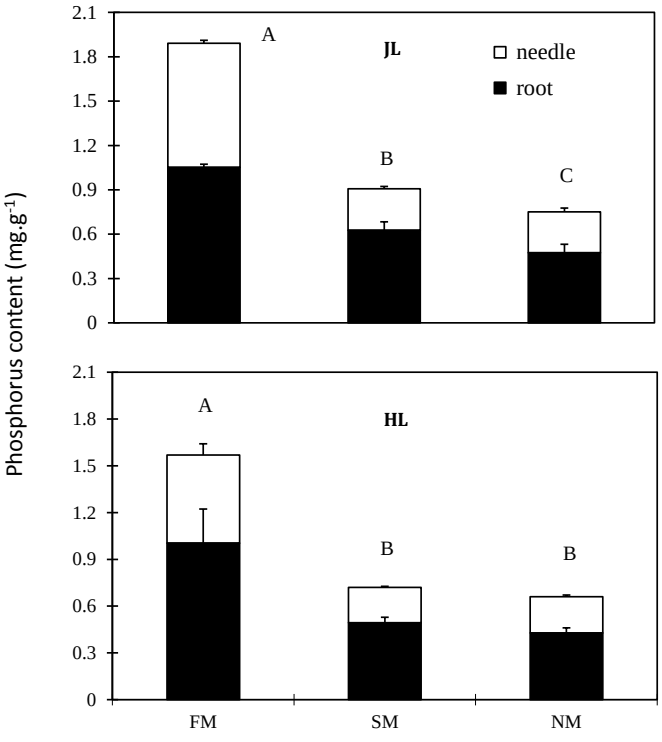

Figure 6.12
The phosphorus content of needles and root of Japanese larch and hybrid larch in the three treatments. FM, SM and NM respectively represent seedlings colonized by larch-forest soil inoculums, *Suillus grevillei*, or no fungi. Data within a series followed by the same letter do not differ significantly ($P<0.05$). Vertical bars represent the standard error of a mean (+SE).

The ectomycorrhizal treatments (EM; FM and SM) had significant effects on P concentration compared to NM seedlings of the Japanese larch. The P of FM seedlings was also significantly different from values for NM seedlings of the hybrid larch ($P<0.05$). However, there were no significant differences between SM and NM treatment on P in the hybrid larch seedlings.

6.3.3 Discussion

In some forests that have infertile conditions growth of conifer seedlings often restricted by the lack of essential mineral nutrients in the soil unless they develop a mycorrhizae symbiosis in their root systems (Trofymow and van den Driessche 1991).Growth of our FM seedlings of both the Japanese larch and hybrid larch was markedly greater than NM seedlings after 110-day colonization. Enhancement of plant growth by mycorrhizal infection is usually caused by an improvement in the nutritional status of the plant or water supply. The higher biomass and N and P contents in FM seedlings (Figures 6.8, 6.12) clearly indicate that ectomycorrhizae enhanced larch growth during the inoculation period. In all treatments the Japanese larch incorporated more ^{14}C and nutrient than the hybrid larch. This may be due to its relative faster rate of growth larch than the hybrid larch. The significantly raised level of phosphorus (P) in FM seedlings suggests that the growth of larch seedlings was limited by the Pavailability.

According to the A-Ci curve, EM seedlings showed greater photosynthetic ability than NM seedlings. This can have a great effect on the growth response curves or fundamentally change the nature of growth response of larch seedlings at the beginning of the growing season. At light saturation and with fully activated enzymes, the initial slope governs the carboxylation capacity of the needle, which in turn depends on the amount of active Rubisco (Lambers *et al*. 1998). The higher CE in EM seedling shows the amount of active Rubisco was larger in NM seedlings, reducing the Pmax in EM Japanese larch seedlings that is normally higher than in NM seedlings.

The maximum photosynthetic rate at high CO_2 concentration indicates the rate of RuBP-regeneration limited region by way of Pi (Sharkey 1985). The supply or utilization of P is considered to be an important factor limiting the value of Pmax. When photosynthesis is limited by trios-phosphate utilization, both Rubisco activity and the rate of RuBP regeneration may be reduced to match the capacity for trios-phosphate utilization (Sharkey, 1985). The reduced phosphorus content in NM seedlings can possibly limit the photosynthesis.

S. grevillei inoculation didn't enhanced larch seedling growth although there was a significantly higher phosphorous content in the Japanese larch and higher photosynthetic rate in both larch seedlings. We suggest there was a possibility that the respiratory of roots acquired larger photosynthate in SM seedlings. Rygiewicz and Andersen (1994) reported total above-ground ^{14}C respiration by mycorrhizal seedlings was about 10% less than non-mycorrhizal seedlings, whereas below-ground respiration of mycorrhizal seedlings was about 35% higher.

Stomatal movements provide the leaf with the opportunity to change the site of carboxylation and the rate of transpiration with the partial pressure of CO_2. In turn, changes in transpiration rate can cause changes in temperature and water potential at the leaf. Stomatal limitation of photosynthesis (Ls) is often thought of as the contribution of stomatal resistance to some total "resistance" to CO_2 uptake (Farquhar and Sharkey 1982). The high Ls in NM seedlings may indicate that a higher resistance to diffusion CO_2 and cause the partial pressure of CO_2 at the sites of carboxylation to be less for EM seedlings. Moreover, high Ls also reduced leaf transpiration in NM seedlings. In other word, EM larch seedlings can survive better in a water shortage, compared to NM seedlings, in view of involvement of ectomycorrhiza in water supply.

C translocation from needles to roots was not enhanced by ectomycorrhiza infection after 24hrsof exposure. It appeared that the infection with ectomycorrhiza did not affect the ^{14}C distribution ratio to roots of larch seedlings. In this case, the absence of significant infection, i.e. non-infection and not fully activated infection conditions, plants may assign larger amounts of photo-assimilates to develop roots or to other activities (such as organic acids synthesis and exudation or enzyme secretion, etc.) in an attempt to overcome the unfavorable conditions.

Competition between the fungus and the roots for photosynthates is the main factor responsible for the typical larger shoot/root ratio in mycorrhizal plants (Berta *et al*. 1990). If incremental C allocated to belowground is a cost of ectomycorrhiza to the plant, and incremental P uptake is its benefit, then growth of FM seedlings proved to be overall enhanced by this lower cost-benefit relation relative to NM seedlings. Movement of nutrients within an ectomycorrhizal mycelial network, as well as exchange of C and nutrients between symbionts, appear to be regulated by source-sink relationships (Simard *et al*. 2002).

Despite significant increases of photosynthetic rate over NM seedlings with greater tissue N and P concentration, there was no accompanying increase in growth of SM seedlings of hybrid larch. This is because stimulation of the photosynthetic rate arising from increases in sink strength is not sufficient to compensate the costs of the production of mycorrhizae and costs associated with extra-radical mycelium (Dosskey *et al*. 1990, Conjeaud *et al*. 1996). The photosynthetic activity is high in FM seedlings of the Japanese larch and hybrid larch, so that they can compensate the lower distribution ratio to the root. This means at under similar conditions a larger amount of photo-assimilates is transported to roots to support the activity of ectomycorrhizae.

Across all treatments, the root/shoot ratio was greater in the hybrid larch than the Japanese larch. That means hybrid larch allocated more resources to root growth than shoot growth during the inoculation. This may be a genetic trait. The mother tree, *L. gmelinii*, is distributed

more in the northern to eastern part of Eurasian continent (Schulze *et al*. 1995) than the Japanese larch. The hybrid larch has a shorter growth period than the Japanese larch and this may be the reason why they allocate more photosynthates to its root.

Irrespective of ectomycorrhiza formation, the root/shoot ratio diminishes during early growth of the young seedlings, and is lower in soil rich in nutrients, especially N; it is also liable to be reduced in conditions where photosynthesis is reduced (Qu *et al*. 2003b). This suggests that the root system of FM seedlings should have greater abilities to absorb water and nutrients, leading to an increased concentration of nutrients in plant tissues, supported by lower stomatal limitation.

Qu *et al*. (2003a) reported, using an *in vitro* synthesis technique, that theJapaneselarch and the hybrid larch seedling have the ability to form ectomycorrhizae with six species of ectomycorrhizal fungi. However, regulation of photosynthates in both species may be different for different ectomycorrhizae. FM seedlings were more markedly enhanced by ectomycorrhizal infection than SM or NM seedlings. There might be connection with the diverse ectomycorrhizal infection with forest-soil inoculums and also ecologically adapted forest-soil inoculums in FM seedlings. The relatively high pH of the standard soil may restrict the activity of *S. grevillei* to form ectomycorrhizae.

In conclusion, this experiment has confirmed using larches which are ectomycorrhizal plants are more efficient than non-ectomycorrhizal plants in growth and in acquiring P and N at the early stage of establishment. Ectomycorrhizal fungi may play an essential role in regulating the rate of carbon or nutrient transfer. They can fundamentally change the nature of photosynthetic response curves, increasing carboxylation efficiency and reducing the stomatal limitation of larch seedlings at the beginning of the growing season.

Chapter 7
Quantitative Estimation Soil Respiration Rateina Larch Forest

7.1 Introduction

Larch plantations have become the principle forest ecosystem of northern Japan. Recently, the natural regeneration of forests has become increasingly important in the conservation of forestland after natural and man-made disturbances. If we are to expect successful natural regeneration of larch stands, we should prepare adequate environmental conditions or regenerated larch seedlings, in relation to light, water, and CO_2 conditions. Several studies have revealed that larch species are light demanding conifer (Gower and Richards 1990, Igarashi *et al*. 1987, Kayama *et al*. 2004). Regenerated larch seedlings in a forest gap or its edges efficiently use light and CO_2 near the ground (Koike *et al*. 2000). In fact, seedlings in a forest promote photosynthetic production by using incidental sunflecks under high CO_2 conditions (Naumburg *et al*. 2001). CO_2 concentration near the ground (soil respiration) varies largely throughout the day and the season (Bazzaz *et al*. 1987).

CO_2 is released from soils in the process variably referred to as either soil respiration, soil-CO_2 evolution, or soil-CO_2 efflux (Raich and Schlesinger, 1992). Forest soil respiration is the sum of heterotrophic (microbes, soil fauna) and autotrophic (root) respiration.

Using a girdling method on the stems, root respiration has recently been estimated, to be nearly 50% of total soil respiration in a boreal Scots pine forest (Högberg 2001). Therefore, it is concluded that soil respiration can be modified by forest harvesting or management. We should analyze the components of soil respiration as resources for CO_2 for regenerated seedlings in order to better manage the stand structure of mature larch forests. Moreover, the extensive larch forests probably play an important role in the global carbon cycle since many studies suggest that the boreal forest in the northern hemisphere is a large CO_2 sink.

The carbon cycle in soil has attracted much attention because it accounts for the second largest flux from terrestrial ecosystems. Raich and Schlesinger (1992) estimated that global soil respiration consists of 50 Pg C • $year^{-1}$ from detritus and 18 Pg C • $year^{-1}$ from live roots and mycorrhizae. The contribution of each group needs to be understood in order to evaluate the implications of environmental changes on soil carbon cycling and sequestration. However, accurate measurements of soil CO_2 efflux are still difficult to obtain. Several methods of estimating soil respiration have been compared in order to obtain a more accurate means of estimation (Liang *et al*. 2004).

Increasing atmospheric CO_2 concentrations have enhanced our need to better understand the source of CO_2 for the photosynthesis of regenerated seedlings and the global sources and sinks of carbon, and their responses to environmental changes. It is necessary to study the components of soil respiration and gain a much better understanding of the factors affecting the rate of soil respiration.

In order to predict the relationship between the source of CO_2 for regenerated seedlings and soil processes, we must first try to understand the relative contributions of root respiration and respiration by soil heterotrophies to total soil respiration.

The fraction of total soil CO_2 efflux derived from live roots is independent of soil C pools, and live root contributions to total soil respiration must be understood before measurements of total soil respiration can be used to infer rates of long-term soil carbon storage (Hanson *et al*. 2000). Therefore, CO_2 released from the roots of standing crops (trees) may be greatly altered by the forest management system, for example, thinning or harvesting of the forest (e.g. Högberg 2001). In addition, microbial communities will be affected if plant inputs, (litter fall), which are the primary source of organic matter for soils, are changed under elevated CO_2 environments, which could have potentially huge impacts on the global carbon cycle.

The objectives of this study are as follows:

(1) To separate the root and microbial contribution to soil CO_2 efflux *in situ*

(2) To evaluate the effects of soil temperature and

soil moisture, soil C and N, and soil microbial biomass on the soil CO_2 efflux of a Japanese larch forest in northern Japan.

7.2 Study site and methods

7.2.1 Study site

This study was carried out in forestry compartments No. 1196 and 1198 of a 50 year-old plantation (as of 2000) of Japanese larch trees in the Tomakomai National Forest, Hokkaido, Japan, from 2001 to 2003. The altitude is 115-140m a. s. l. The tree cover at the site is predominantly Japanese larch, interspersed with Yezospruce (*Piceajezoensis* Sieb. et Zucc.) and mixed broadleaved species (birch, oak, magnolia etc.). In 1999, the overstory density was about 1087 stems ha^{-1}, the total basal area was about 23.5m^2 ha^{-1} and aboveground biomass averaged 145m^3 ha^{-1}. The average tree height was 18~20m, in 2000. The forest canopy had a mean depth of 8.9m and leaf area index (LAI; m^2 projected tree leaf area m^{-2} ground area) of approximately 2.0. The forest understory was predominantly buckler fern (*Dryopteris crassirhizoma*), with occasional bracken (*Dryopteris expansa*) and Japanese spurge (*Pachysandra terminalis* Sieb. et Zucc.).

The site is characterized by a humid continental climate with cold winters and cool summers, but with no predominantly wet or dry season. Mean annual precipitation at the site is approximately 1250 mm, and the mean annual temperature is 7.3°C, with a variation in the monthly mean value ranging from 19.1°C in August to –3.2°C in January. The site has an essentially flat topography, with a gentle slope varying by a 1~2 degree gradient. Geologically, the soil at the site is homogeneous, with a well-drained arenaceous soil derived from volcanic ash, and is classified as immature Volcanogenous Regosols (Pumice). The soil pH ranges to 5.0 to 6.0 and the nutrient level is poor with a high porosity. The litter layer has a thickness of 1 to 2cm. The estimated root biomass at 13.1 Mg ha^{-1} was mainly confined to a narrow soil zone (10~15cm) between the overlying layer of litter and the underlying, water-deficient, porous pumice (Sakai *et al*. 2001).

7.2.2 Trenching method

Trenching with root exclusion is a straightforward approach to measuring soil respiration without roots on relatively undisturbed soil using standard surface flux techniques. Eight 0.6m (L) × 0.6m (W) × 0.45m (H) plots were set up in this Japanese larch forest in May, 2001. Roots were excluded using corrugated plastic sheets. The litter layer on the forest floor was cleaned for four of the plots. Measurements were made from June 2001 to Dec. 2003 in order to determine the seasonal changes of root and microbial respiration.

7.2.3 Measurements

The respiration rate of the soil was measured using a LI-6400 portable photosynthesis system (LiCor, Inc., Lincoln, NE, USA). LI-6400 can be used for measuring the soil-surface CO_2 efflux when fitted with a null balance soil chamber (LI-6400-09). The measurement errors associated with the disturbance of the soil and roots were minimized by using permanently inserted collars, 3 to 4cm into the soil, as an interface between the soil and the chamber. All vegetation was removed from inside the collars. The mixed air in the chamber was withdrawn at the top of the chamber through the analyzer, which was attached directly to the chamber. Air was returned from the analyzer to the chamber through a manifold near the soil surface.

The soil CO_2 efflux was calculated from the increase in CO_2 concentration over time, the volume of the entire system (991cm^3) and the enclosed soil surface area (71.6cm^2). The closed chamber employed a pressure equilibration tube, which eliminated the effect of chamber pressurization on measured CO_2 efflux. Normally, the measurements started at 12:30 and ended at 16:00. According previous study of Yanagihara (2001), she measured 100 points within a 20m × 20m site. There was small variation in some places. We used the average of the measurements across all 20 points for analysis. Soil temperature was concurrently measured with an attached soil temperature probe at about a depth of 5cm. The water at the soil core was sampled each time the respiration was measured, using the core sampler (DAIKI Co., Ltd.) (Volume is 100cm^3). Soil microorganism biomass (C and N) was determined by using the method described by Brookes *et al*. (1985). Soil C and N were analyzed by using an N-C analyzer (NC900, Shimadzu, Osaka, Japan).

7.2.4 Data analysis

The root respiration (R_r), heterotrophic respiration (R_h) and litter respiration (R_L) were calculated using the following equations:

$$R_r = R_s - R_h,$$
$$R_L = R_h - R_l,$$

where R_s is the total soil respiration in the control plot, R_h is the heterotrophic respiration in the trenched plots coving the litter. R_l is the soil respiration in the trenched plots without covering litter. Volumetric water content (%) was calculated using the equation as follows,

Water content (%) = 100 × (fresh soil – dry soil)/fresh soil,

where fresh soil is the weight of soil (g • m^{-3}) samples from the field, dry soil is the weight (g • m^{-3}) of soil dried in the oven by 60°C, 24hr.

7.3 Results

7.3.1 Seasonal changes in soil respiration rates, soil temperature

The soil temperatures at a depth of 5cm, from May through October of 1st to 3rd year after trenching applied, are shown in Figure 7.1. Soil temperature increased steadily until mid-summer, reaching a maximum in 1st year of 19.4 °C on August 23rd, of 19.6°C in 3rd year on August 10th and in 2nd year of 19.8 °C on September 3rd. From this peak the soil temperature declined to the end of October in each of the three years. Mean growing season soil temperatures (at 5 cm depth) are shown in

Figure 7.1.This shows a very clear seasonal change pattern, which is similar to that of the soil respiration. The maximum soil temperature at 5cm was approximately 20°C in early August during each of the three years. Moreover, there was not a large temporal variation of the soil temperature at 5cm depth. There was a significant exponential relationship (r^2=0.81, P<0.05, n=21, each value is the mean value of every month) between soil respiration and soil temperature (Figure 7.2).

Volumetric soil water content did not show a significant seasonal change (data not shown).

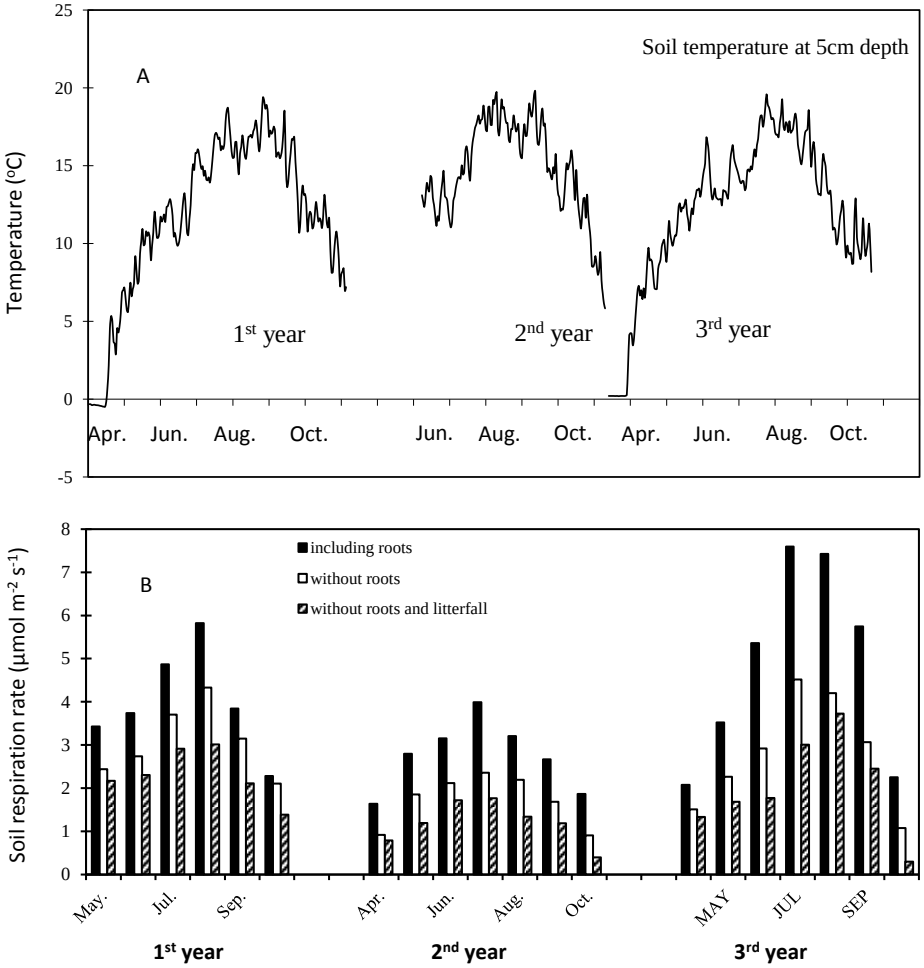

Figure 7.1 A: The daily mean temperature of soil at 5cm depth in the Tomakomai National Forest from April to October in 2nd and 3rd year and from June to October in 1st year
B: Monthly changes in soil respiration rates in "no-roots" plots, "no-roots-no-litterfall" plots and control plots from 1st to 3rd year.

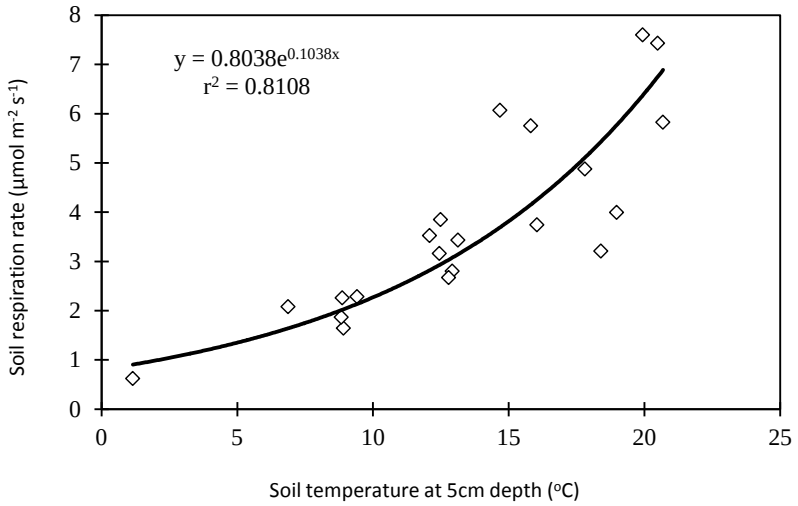

Figure 7.2 The exponential relationship between soil respiration rate and soil temperature at 5cm depth. Data were mean values of each month, n=21.

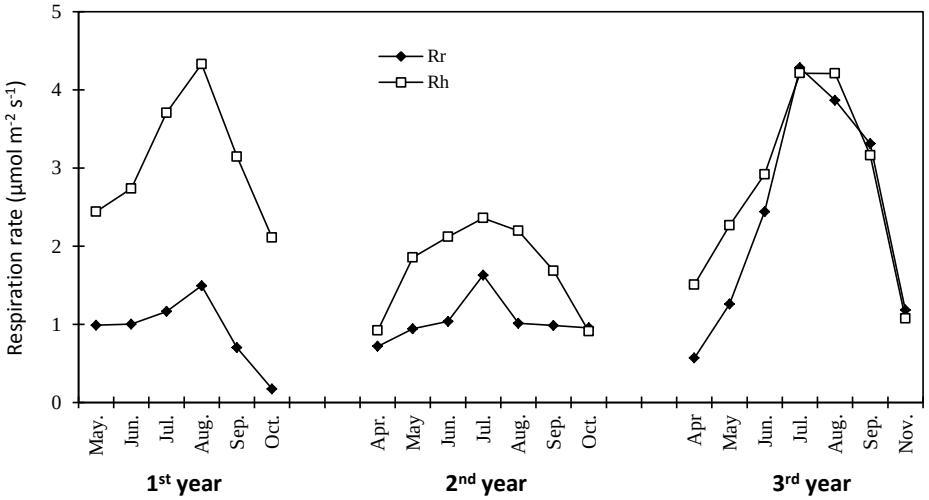

Figure 7.3 Monthly changes of root and heterotrophic respiration rates in 1st, 2nd and 3rd year.

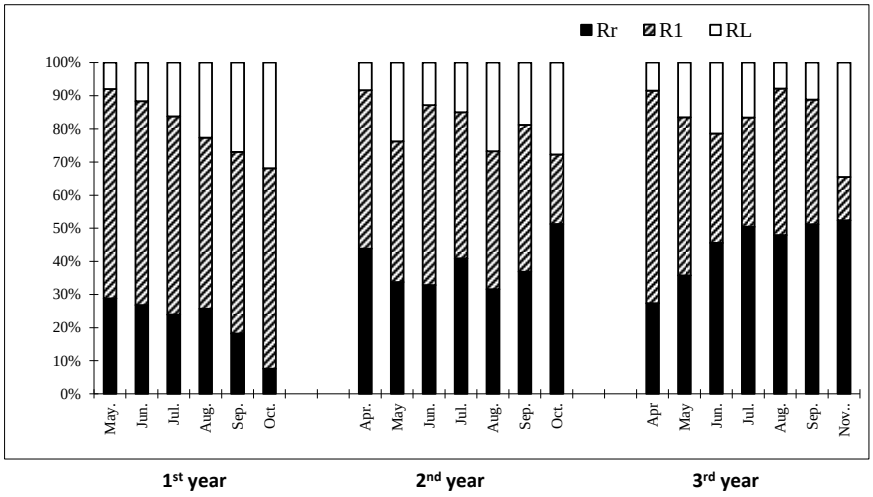

Figure 7.4 The percentage of root respiration (Rr), respiration from the trenched plots without coving litterfall (R1) and litter respiration (RL) in 1st, 2nd and 3rd year.

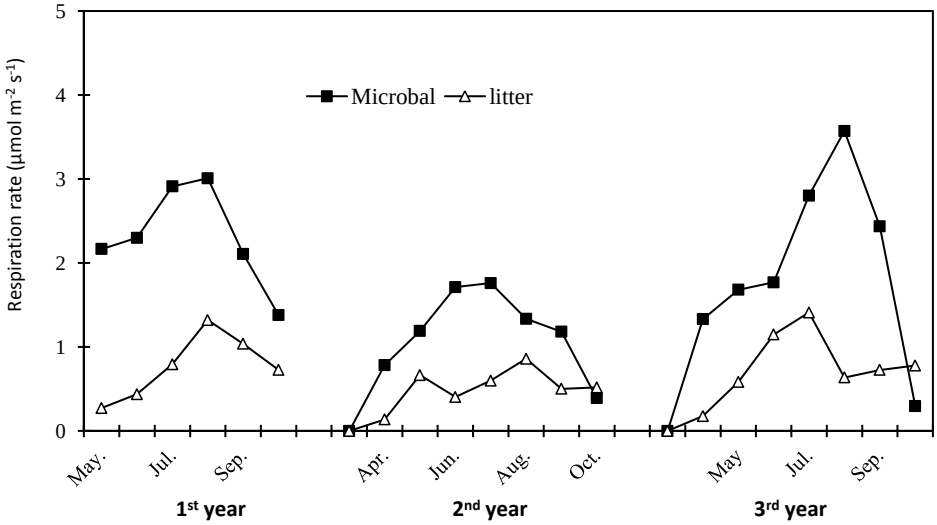

Figure 7.5 Monthly changes of soil microbial respiration and soil respiration from litterfall.

7.3.2 Estimation of root respiration and soil microbial respiration

Root respiration (R_r) to the total respiration in 1st year ranged from 18-28%. It ranged from 32 to 51% in 2nd year and from 27% to 52% in 3rd year. The pattern of root respiration changed seasonally (Figure 7.3). Rr increased toward August then decreased from September.

The heterotrophic respiration shows a very similar pattern of seasonal change to that of root respiration during the growing seasons from 2001 to 2003 (Figure 7.3). The contribution of heterotrophic respiration (R_h) to soil respiration in 1st year was estimated to be from 70–80%, and 49–68% and 48–72% for 2ndand 3rd year, respectively. It is apparently larger than the root respiration rate in 1st year and 2nd year, and nearly equal to the root respiration rate of 3rd year. The respiration rate from the litter decomposer (R_L) was lower than that of from the soil microorganism (Figure 7.5). The percentage of each component (root, soil microorganism and litter fall) to soil respiration was estimated and shown in Figure 7.4. Root respiration accounted for the majority of soil respiration in 2nd year and 3rd year. The lower respiration in 1st year compared to 2ndand 3rd year may be due to the soil disturbance during the first trenching year. The contribution of litter respiration to soil respiration ranged from 8% to 32% during the three years. The lowest value (8%) was measured in April of each year (Figure 7.4).

7.3.3 Seasonal pattern of soil microorganism activities

Soil microorganism biomass carbon (MBC) and soil microorganism biomass nitrogen (MBN) had a positive linear relationship (r^2=0.75, n=19, n is the mean value of every month) in 1st, 2ndand 3rd year (Figure 7.8). There were apparently positive relationships between MBN, MBC and volumetric water content (Figure 7.6, 7.7). This trend was very similar in 1st year and 3rd year, but showed a slight variation in 2nd year.

Soil C and soil N also had a close linear relationship (r^2=0.96, n=19, n is the same soil sample used for analyzing the soil microorganism in 2003) (Figure 7.9). The soil microorganism biomass closely resembled soil C and soil N (Figure 7.10), however, it did not resemble the soil heterotrophic respiration rate.

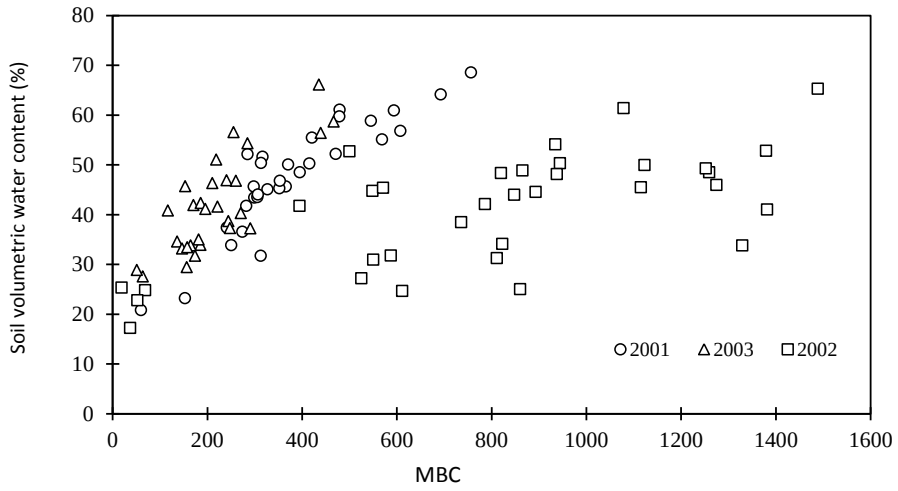

Figure 7.6 The relationship between soil volumetric water content (%) and soil microorganism biomass carbon (MBC). Data were mean values of each month during three years, n=21.

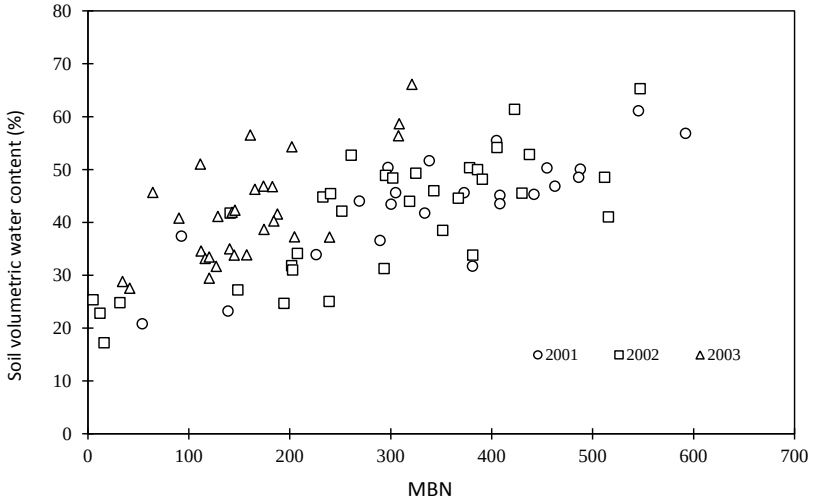

Figure 7.7 The relationship between soil volumetric water content (%) and soil microorganism biomass nitrogen (MBN). Data were mean values of each month during three years, n=21.

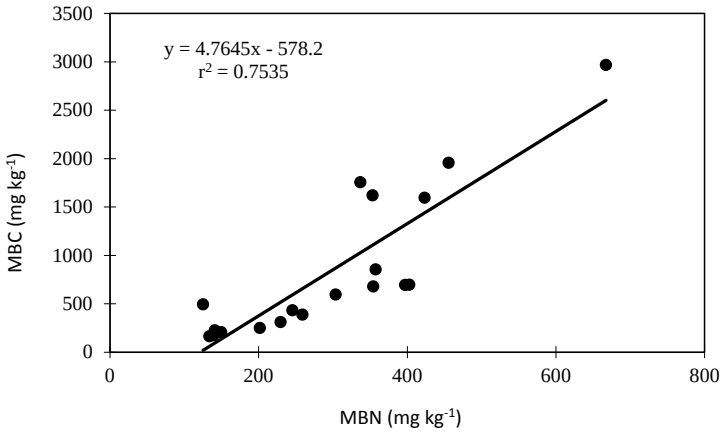

Figure 7.8 The linear relationship between soil microorganism biomass carbon (MBC) and soil microorganism biomass nitrogen (MBN). Data were mean values of each month during three years, n=21.

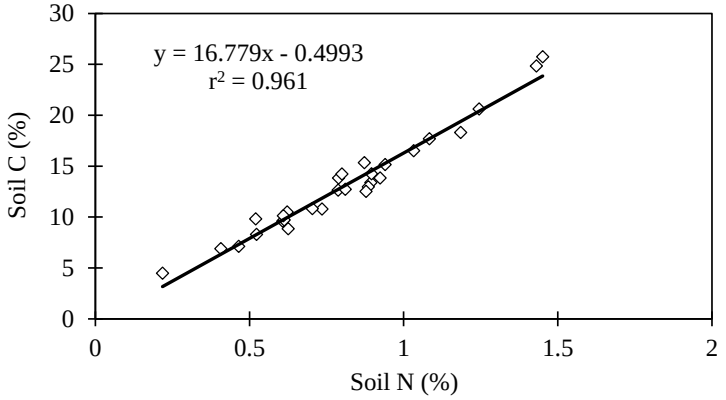

Figure 7.9 The linear relationship between soil carbon and soil nitrogen in 3rd year, n=28.

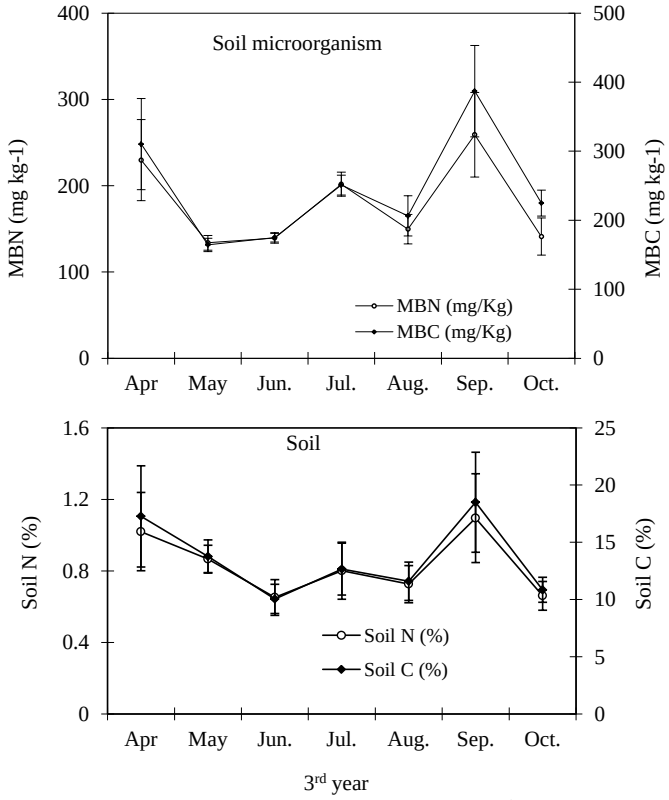

Figure 7.10 Monthly changes of soil microorganism biomass (carbon and nitrogen) and soil carbon and soil nitrogen in 3rd year.

7.4 Discussion

Since much of respiration occurs in the upper organic layers, temperature changes can strongly affect respiration rates (Figure 7.1). The soil temperature is the most important factor in regulating the soil respiration and is also the most intensively studied factor (Lin *et al*. 1999, Luo *et al*. 2001, Winkler *et al*. 1996). Our data also showed that soil respiration increases exponentially with increasing temperature. The seasonal change of root respiration may have been due to increased root respiration resulting from the active root growth, associated with above-average production from April through July, warm soil temperatures and a peak in larch growth.

One of the biggest concerns with the trenching approach is the influence of residual roots that are left and decompose in the trenched plots and their contribution to the total soil respiration. In 2001, the Rr in the trenched plots was estimated to be from 18–28% and R_h was estimated to be 70–80%. This may be due to the soil disturbances of the current year. In 2nd and 3rd year, the R_r ranged from 30 to 50%.There was no big variance within these two years. We assumed residual root decomposition contributed little to underground respiration, and there was no soil disturbance after the plots had been trenched for one year. Previous studies (Lee *et al*. 2003) clearly suggest that all root exclusion approaches that disturb the natural soil profile need to time for re-equilibration in order to steady the state conditions and to minimize the impact created by disturbance artifacts. Buchmann (2000) ran a series of root exclusion experiments with staggered start times, allowing for the comparison between recently cut roots and roots that had been dead for up to six months.

Soil moisture is another important factor influencing soil respiration. Soil CO_2 efflux is usually low under dry conditions due to low root and microbial activities, and increases with soil moisture until a certain limit. In very high soil moisture conditions, soil CO_2 efflux is reduced due to the limitation of the diffusion of oxygen and the suppression of CO_2 emissions. The relationship between soil respiration and moisture is usually scattered (in other words quadratic, linear, exponential and hyperbolic equations) and our understanding of this relationship and the mechanisms underlying the relationship is still limited compared to that of the respiration/temperature relationship. According to our results, soil volumetric water content shows a close relationship with the soil microorganism biomass (Figure 7.6, 7.7). This indicates that soil water is an important factor affecting the soil microorganism growth. However, the soil water content had a very small affect on soil respiration, which may be due to the small change in water content in the soil. The soil microorganism biomass was also passive in soils C and N, where it is usually considered to be their food.

There was no relationship between soil microorganism biomass and R_h. In the field, there are many kinds of soil micro-organisms, however, it may be that not all types of soil microorganisms are active during the growing season. Only parts of the soil microorganisms were very active in the spring, summer and autumn. Their activities may be closely related to the R_h. The analysis of the soil microorganism activity *in situ* will be needed in future research.

The contribution of R_h to soil respiration was shown to be more than 50%. However, this may be an overestimation. Hanson *et al*. (2000) suggest that, when transpiration ceases after root exclusion, soil moisture may increase, which ultimately leads to altered heterotrophic respiration rates, depending upon the preexisting soil moisture conditions. The increasing soil moisture can affect not only the heterotrophic respiration rates but also the decomposition rate. Moreover, organic matter around the rhizosphere (including fine roots, mycorrhizae, soil microorganisms, and labile organic matter) continues to contribute to surface flux shortly after killing the root (< 1 month).

The contribution of respiration from litter to soil respiration ranged from 8–32%, which indicated that the litter decomposition was an important factor in the soil CO_2 flux. This ratio was at its lowest in April and increased with the soil temperature. This demonstrates that soil temperature is the key factor during the litter decomposition.

Högberg (2001) reported tree-girdling in *Picea abies* trees reduced soil respiration within 1–2 months by about 54%. He showed the flux of current assimilates to roots was a key driver of soil respiration. Thus, forest management can modify the soil respiration. Analyses of the components of soil respiration as CO_2 resources were also important for regenerated seedlings in the forest floor.

Chapter 8

General Discussions

8.1. Natural regeneration of larch forests

The larch species (*Larix* spp.) is one of the most important conifers for both biological resources and the conservation of nature because of its broad distribution in the northern hemisphere. In particular the *L. gmelinii*, which covers the permafrost region of northeast Eurasia (mainly Sakha Republic) (Schulze *et al*. 1995). Sakha people in Russian Federation have been conserving the larch forests using a selective cutting method in order to keep it as natural as possible. These regions are also believed to help moderate global warming by the larch species CO_2 fixation.

Japanese larch (JL) is one of the most important plantation species in northern Japan because of its high growth rate for timber production--namely its high CO_2 fixation capacity. However, it has been suffering from shoot blight disease and grazing damage done by voles partly because it is not native to Hokkaido, but originates mainly from central Japan and was introduced into northern Japan during the early 1900's. Therefore, hybrid larch trees F_1 (HL: *L. gmelinii*var. *Japonica*x *L. kaempferi*) were developed in order to protect the trees against these biological damages. The mother trees (*L. gmelinii*) originated from the Kurile Islands. Now, larch plantations cover approximately 4700km^2 of Hokkaido and have become a principle forest ecosystem of northern Japan. However, the more recently planted HL plantations are also reaching the

stage of seed production.

Recently, we have come to expect that forests should have several functions, not only timber production but also the conservation of forestlands, water quality, recreation, and habitat for wildlife. It is expected that the natural regeneration of both larch species after selective cutting and disturbances, such as fire or volcanic eruptions will help to maintain these functions within the forest. Several studies on the analysis of the natural regeneration processes of larch forests were performed in Hokkaido (e.g. Sasaoka *et al*. 2000, Yajima 2000) and throughout Japan (Igarashi *et al*. 1987). Based on these vegetation studies, the JL is a typical, light-demanding, early successional type of species and has established itself in open spaces after harvesting, naked areas produced by landslides, and after volcanic eruptions.

In general, successful regeneration of a tree species is closely correlated with natural conditions, such as the micro-environment and the growth characteristics of the species. If the physical conditions match the growth characteristics of the plants, then the seeds and their seedlings will establish themselves under competitive conditions with other species (in other words, other plants, microorganisms, macro-fauna, etc.). Therefore, in order to promote the natural regeneration of JL and HL forests, we should try to understand both the habitat and the growth traits of both species. Both species havetypical light demanding characteristics, and therefore, high interspecific competition between JL and HL during the stage natural regeneration of the seedlings is predicted to exist. The competition is usually very strong between closely related species. To provide basic information for natural regeneration of the JL and HL, this study focused on the soil environment's affect on the regeneration of the two species and aimed to investigate how the environmental factors influence the ecophysiological characteristics of larch growth.

Based upon ecophysiological field studies and experiments under regulated conditions, we concluded that the growth responses of the Japanese larch seedlings and the hybrid larch seedlings were slightly different under the same environmental factors, such as light flux, soil temperature, and soil fertility (Table 8.1).

The traits of the two species shown in Table 8.1 are relatively expressed between JL and HL. In practical forestry, we have been using HL as F1 larch for approximately 40 years. Moreover, the Hokkaido regional government decided to introduce HL for creating plantations with a higher CO_2 fixation capacity. The commercial name of HL is “Greem” (taken from “green” and “dream”) because HL has a relatively high growth rate, higher resistant traits against several biological stresses and a higher density of xylem (Hokkaido Regional Government 1987, Kuromaru 1995). As a result, it is hoped to soon have HL seedlings in the stage of natural regeneration.

As listed in Table 8.1, it has been demonstrated that HL can uptake the nitrogen more efficiently than JL. Soil temperatures above 7ºC, are considered to be essential for the growth of JL. In contrast, F1 can adapt to low soil temperatures and low nutrient conditions better than JL. Although the species of ectomycorrhiza has not yet been identified, the allocation of photosynthates of HL was larger than that of JL. According to these allocation traits in HL, we should pay attention to forming a symbiotic relationship between HL and ectomycorrhiza. Under shaded conditions, HL may have a high capacity for reducing nitrate nitrogen to ammonium nitrogen. Therefore, it is expected that HL in fertile nitrogen conditions may be able to survive under shaded conditions better than JL. Since the development of the root is plastic (Fitter *et al*. 1998), the establishment of a root system is very important in order for seedlings to regenerate. Lacks of light and lower soil temperatures markedly reduce the root growth of both larch species. Moreover, the two larch species are sensitive to fertilization during nursery culture. Different conditions of soil fertility affect the root distribution of JL and HL seedlings.

Our results indicate that the ectomycorrhizal seedlings of both larch species were more efficient than the non-ectomycorrhizal seedlings, in growth and in acquiring phosphate and nitrogen at the early stages of establishment. The ectomycorrhizae benefited the growth of both larch species. Dry mass, concentration of nitrogen, and phosphorus in ectomycorrhizal seedlings were significantly higher than in non-ectomycorrhizal seedlings. These data suggested that infection with ectomycorrhizae seems to be beneficial for the establishment of regenerated larch seedlings of both species with the natural existence of charcoal (Makoto *et al*. 2012).

After forest fires, charcoal provides for a different type of relationship between the ectomycorrhiza and the seedlings (Wardle *et al*. 1988, Pietikäinen *et al*. 2000). In this sense, we should learn more about their

Table 8.1 Summary of the growth characteristics of the seedlings of the two larch species in fields and laboratory conditions

Environment / species	Light condition	Soil temperature	Nutrient or soil fertility	Ectomycorrhiza symbiosis
Japanese larch	Higher light dependency	Narrower growth range	High nutrient uptake ability	Higher infection rate
Hybrid larch	Lower light dependency	Adapt lower soil temperature of ca. 7°C	Lower nutrient uptake ability	Lower infection rate

interactions in regard to the establishment of regenerated larch seedlings and symbiotic microorganisms.

In all, under relatively better growth conditions (for example full sunlight, 15°C soil temperature etc.), the Japanese larch seedlings have a greater growth rate (allocate more biomass and nutrients) than that of the hybrid larch. This has been examined by Koike (2000) and Ryu *et al*. (2009). However, we think this just happened under favorable environmental condition for JL. Otherwise, HL will be more competitive than JL, such as in cold regions (i.e. northern Japan) or higher regions since HL will be better able to grow under a low or wide variation in soil temperature. Given the slight shade condition such as at a gap in the forest, and fertile soil condition, HL may also dominate since the growth of JL was clear suppressed under such situation. Moreover, HL was introduced to protect again some biological damages, which suffered by JL. It also makes HL more competitive than JL.

8.2. Contribution of forest rehabilitation practices

In China, a half-century old policy of forest exploitation and monoculture has led to disastrous consequences, including the degradation of forests and landscapes, the loss of biodiversity, unacceptable levels of soil erosion, and catastrophic flooding. To conserve the natural forests, the Chinese Government has adopted a new forestry policy (The Natural Forest Conservation Program (NECP)), which emphasizes the expansion of natural forests and increasing the productivity of forest plantations (Zhang *et al*. 2000). Fast-growing tree species, such as larch, birch and popular were promoted for planting. However, they did not pay much attention to the method of biodiversity conservation or regeneration of the seedlings in forest plantations after excessive cutting.

If we are to have successful plantations and natural regeneration, we should know the environmental capacity of the target area, such as the light conditions, temperature, and nutrient conditions. We should also know the conservation techniques of biodiversity in genetics, species and landscape levels (Frankel *et al*. 1995). According to this study, the ecophysiological research on natural regeneration also provides some useful information for forest research, especially in northeast China, where they have very similar plant vegetation to Hokkaido (Shi *et al*. 2001). With an increasing number of species in a given area, the net primary productivity increases asymptotically to some extent (Chapin *et al*. 1998). Larch forests usually allow several types of undergrowth because of their relatively sparse canopy, especially in Japanese larch forests. Therefore, we should think about tending methods for maintaining a high productivity for CO_2 fixation.

Moreover, preparing seedling stocks in the nursery is also important for a plantation. An exponential delivery schedule is an efficient fertilization technique for greater nutrient uptake of plants because it increases or maintains the N concentration of the whole plant. This technique has been shown to be a very efficient fertilization technique for many tree species, for example, JL and HL, Chinese fir, and black spruce (Quoreshi and Timmer 2000). So far, there has been a lack of use of exponential techniques in order to produce seedlings in the nursery culture in northeastern China. This practice should be introduced in order to promote the healthy growth of forest plantations in the future. Moreover, the important role of ectomycorrhiza symbiosis on the Silviculture also should play an essential role in establishing seedlings under new conditions. Ectomycorrhizae can be found in about 90% of the trees in temperate and boreal forests. The ectomycorrhizal inoculation is beneficial after transplanting.

The most important factor influencing the success or failure is the vigor of the natural ectomycorrhizal population. By using the forest-soil inoculum in our experiment, we discovered that there may be a better way to promote the establishment of ectomycorrhizal larch seedlings in nature. In general, the newly introduced fungi have to adapt to the ecological conditions of the reforestation sites—climate, soil pH, fertility and other components of the ecosystems, in particular the native vegetation.

However, the inoculated ectomycorrhiza does not always harmonize with original fungus flora. The techniques for the production of tree seedlings infected with competitive and efficient ectomycorrhizal fungi are not enough. New methods, such as pyrosequencing and meta-genomic approach could become powerful tools for identifying fungal species or fungal strains associated with their hosts in field situations. There are still large gaps to be overcome by using ecological, physiological and molecular biological approaches.

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