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# Growth and N<sub>2</sub> fixation in an *Alnus hirsuta* (Turcz.) var. *sibirica* stand in Japan

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To estimate the N<sub>2</sub> fixation ability of the alder (*Alnus hirsuta* (Turcz.) var. *sibirica*), we examined the seasonal variation in nitrogenase activity of nodules using the acetylene reduction method in an 18-year-old stand naturally regenerated after disturbance by road construction in Japan. To evaluate the contribution of N<sub>2</sub> fixation to the nitrogen (N) economy in this alder stand, we also measured the phenology of the alder, the litterfall, the decomposition rate of the leaf litter, and N accumulation in the soil. The acetylene reduction activity per unit nodule mass (ARA) under field conditions appeared after bud break, peaked the maximum in midsummer after full expansion of the leaves, and disappeared after all leaves had fallen. There was no consistent correlation between ARA and tree size (dbh). The amount of N<sub>2</sub> fixed in this alder stand was estimated at 56.4 kg ha<sup>-1</sup> year<sup>-1</sup> when a theoretical molar ratio of 3 was used to convert the amount of reduced acetylene to the amount of fixed N<sub>2</sub>. This amount of N<sub>2</sub> fixation corresponded to the 66.4% of N in the leaf litter produced in a year. These results suggested that N<sub>2</sub> fixation still contributed to the large portion of N economy in this alder stand.

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## 1. Introduction

Biological N<sub>2</sub> fixation is an important pathway for the input of new N to terrestrial ecosystems (Vitousek and Walker 1987; Van Cleve *et al.* 1991). Actinorhizal *Alnus* species that fix N<sub>2</sub> through the metabolic activity of the filamentous bacterial symbiont *Frankia* play an important role in the nitrogen (N) cycle of temperate forest ecosystems (Huss-Danell 1997; Dawson 2008). The range of N<sub>2</sub> fixation rates reported for *Frankia-Alnus* symbiosis coincides with those for *Rhizobium-Legume* symbioses (Huss-Danell 1990; Hibbs and Cromack 1990). These *Frankia-Alnus* systems have been utilized to revegetate deteriorated wildlife habitats and rehabilitate N-deficient disturbed areas (Enoki *et al.* 1997;

Sharma *et al.* 2002; Yamanaka *et al.* 2005; Son *et al.* 2007). In addition, these *Frankia-Alnus* systems have become the focus of interest in responses to elevated atmospheric CO<sub>2</sub> concentrations (Hungate *et al.* 2003; Tobita *et al.* 2005, 2011; Eguchi *et al.* 2008). Globally, there are 47 species of genus *Alnus* (Pawlowski and Newton 2008), 16 of which grow naturally in Japan (Uemura and Sato 1975). However, the information on N<sub>2</sub> fixation in the *Alnus* stand is lacking in Japan.

Wide-ranging of environmental conditions, such as light, soil temperature, water conditions, mineral nutrition, and pH, also affect N<sub>2</sub> fixation activity (Huss-Danell 1997; Pawlowski and Newton 2008). The decline in N<sub>2</sub> fixation activity of nodules caused by these factors is often related to a deficiency of carbohydrates supplied from the leaves to the nodules,

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because N<sub>2</sub> fixation activity depends on newly formed photosynthates transported from the leaves of host plants to the root nodules (Huss-Danell 1997; Valverde and Wall 2003).

In northern temperate forests, actinorhizal plants are usually deciduous tree or shrub species, and nitrogenase activity, which usually begins shortly after leaf emergence in spring, remains high, although varying in summer, decreasing in autumn, and ceasing when all leaves are shed (Schwintzer *et al.* 1982; Huss-Danell 1990; Tsutsumi *et al.* 1993). As mentioned above, N<sub>2</sub> fixing activity depends on newly formed photosynthates transported from the leaves to the root nodules (Wheeler 1971) and the N<sub>2</sub>-fixing root nodules can be regarded as a strong metabolic 'sink', competing with developing leaves, roots and fruit etc. for photosynthetic products within a plant (Huss-Danell and Sellstedt 1983). The growth pattern of alder, such as shoot elongation, flowering and fruiting, will affect the seasonal variation of N<sub>2</sub> fixation through competition using photosynthates (Pizelle 1984).

The nitrogenase activity of each nodule and the N<sub>2</sub> fixation rate per plant will also vary depending on the variation in demand for N with increasing tree age (Sharma and Ambasth 1988; Sharma *et al.* 2002; Son *et al.* 2007). The contribution of N<sub>2</sub> fixation to the N economy changed with stand age, peaking in the 15- to 20-year-old stand of *Alnus nepalensis* (Sharma and Ambasth 1988; Sharma *et al.* 2002).

The nodules of actinorhizal plants, such as *Alnus* species, are perennial (Baker and Schwintzer 1990; Huss-Danell 1997). In a naturally regenerated *Alnus hirsuta* var. *sibirica* stand on the roadside in the central part of Japan, we found as well as the nodule biomass per tree, the nodule size distribution, as presented by the root diameter at nodule base, also changed among tree size (Tobita *et al.* 2010). In such a naturally regenerated stand, unlike plantations, it may be necessary to consider the effects of the *Alnus* species tree size on N<sub>2</sub> fixation activity when we will evaluate the amount of N<sub>2</sub> fixation in stand level, because the nodule age and size will affect the nitrogenase activity of nodules (Sharma and Ambasth 1984; Huss-Danell 1990; Hurd *et al.* 2001)

The N input into soil through the N<sub>2</sub> fixation of actinorhizal plants will boost N accumulation in soil (van Cleve *et al.* 1993; Myrold and Huss-Danell 2003), and enhance the N content of needles and the growth rate of conifer trees, which were mixed-planted with *Alnus* species (Vogel and Gower 1998; Brockley and Sanborn 2003). In addition to the N<sub>2</sub> fixation ability, the high N content of the leaf litter of actinorhizal plants (Huss-Danell 1997), their fast decomposition rate (Osono and Takeda 2004), and herbivorous insects flux (Meehan and Lindroth 2007) will contribute to ensuring an effective N supply into forest ecosystems.

The objectives of this study are to evaluate the amount of N<sub>2</sub> fixation in the 18-year-old *Alnus hirsuta* var. *sibirica* stand regenerated naturally on areas degraded by road construction in Takayama, central Japan, and the contribution

ratio to N cycle in the stand. We try to clarify whether the amount of N<sub>2</sub> fixation differs among tree size, not in terms of the difference in N<sub>2</sub> fixing activity of nodule, but also the difference in nodule biomass per tree, and also whether the amount of N<sub>2</sub> fixation by *A. hirsuta* var. *sibirica* still significantly contributes to N economy in this stand. To test these hypotheses, we examined the phenology, seasonal variation in the nitrogenase activity of nodules, and the amount of N<sub>2</sub> fixation in this alder stand. We also try to evaluate the N<sub>2</sub> fixation rate and contribution ratio to N flow and accumulation rate in this stand.

## 2. Materials and methods

### 2.1 Study site

This study was conducted from 1995 to 1996. The study site was at an altitude of approximately 1100 m on Mt. Norikura in the eastern part of Takayama city, Gifu prefecture, central Japan (36°9'N, 137°15'E). A study plot of 30 × 35 m was set up in an alder (*Alnus hirsuta* Turcz. var. *sibirica* (Fischer) C.K. Schn.) stand as described in detail in Tobita *et al.* (2010). Alders are deciduous, early successional species that are widely distributed in the northern districts and highlands of Japan. Alder regenerated naturally after disturbance by road construction work in 1975. In our study site, all the canopy trees were alders. The tree height was about 15 m, and the canopy of this stand was almost closed (Hasegawa and Takeda 2001). The tree stem diameter was also measured at 1.3 m above ground-line (dbh). The mean (±SD) dbh of trees was 12.4 (±3.8) cm in April 1995 (Tobita *et al.* 2010). The stand density was 1114 ha<sup>-1</sup>, while total basal area was 14.8 m<sup>2</sup> ha<sup>-1</sup> in April. Although the site floor was densely covered in herbaceous plants, regenerating specimens of several species of trees and shrubs, non-N<sub>2</sub>-fixers, were also present. Because several trees died during the study, the stand density changed to 1038 ha<sup>-1</sup> in May 1996. The neighboring forest stand has been used for coppicing, and is now dominated by *Pinus thunbergii*, *Quercus mongolica*, *Betula platyphylla* var. *japonica*, *Prunus grayana*, *Lindera obtusiloba* and *Euptelea polyandra*. The mean annual temperature was 7.1°C, and mean annual precipitation was 2190 mm during the period 1990 to 1995 at an altitude of 1340 m near the study site (data from the Institute for Basin Ecosystem Studies, Gifu University, Gifu, Japan). There was no marked seasonal pattern in precipitation, but the monthly amount of precipitation during June to September differed between the 2 years (1995–1996). In 1995, the monthly amount of precipitation was large in July (395 mm) and was small in June and August (119–134.5 mm). On the other hand, in 1996, it was small in July (88 mm) and was large in June and August to September

(210–294.5 mm). Soil temperature was measured by a data logger (Grant SQ8-4U, Cambridge, England) at depths of 10 and 20 cm respectively every 30 min throughout the growing season.

## 2.2 Growth pattern

Shoot phenology was observed in 93 current shoots (which emerged from 16 one-year-old shoots within 4 two-year-old shoots) selected from two trees at the stand edge from May to November 1996. These shoots were measured once a month. The following events were monitored: length of each current shoot, the number and size (length and width) of all emerged leaves on each current shoot and the number and length of male and female flowers (Hasegawa and Takeda 2001). To convert the size (length by width) of leaves into the leaf area, the size and leaf area of all leaves in shoots of about 30 cm in length were measured once a month from June to September. Each value was shown based on the value relative to the maximum value at the end of the growth period in November. To calculate the attached leaf area, the total leaf area of all leaves having emerged till November was used as the maximum value. In this study, we assumed the seasonal change in phenology of the current shoots was unchanged over the 2 years of measurement. Kikuzawa (1978) suggested that the leaf development and defoliation were specific per tree species, though the mean number and area of leaves per current shoot varied based on the method selected to observe the twigs and probably differed over the 2 years. In this study, we observed several limbs to consider the variation among current shoots. We also assumed the seasonal change in the phenology of the current shoots of sampled trees was representative of the phenology of the whole crown in this stand. As we described later, we estimated the seasonal change of the attached leaf area in the canopy of alder by utilizing these data of shoot phenology and leaf litterfall by litter traps. The diameters at breast height (1.3 m, dbh) were measured for all trees in this study plot every month during growth periods. A regression formula for the growth pattern of dbh and each organ was calculated using a simple logistic model:

$$D_t = D_{\max} / (1 + \exp(-(t - t_m))), \quad (1)$$

where  $D_{\max}$  is the maximum value of each parameter,  $t$  is the day (DOY), and  $t_m$  is the day when the growth rate of each parameter peaks. The growth of nodules was estimated by repeated measurements of nodule lobe length, while the nodules of three trees ( $n = 156$ ) were excavated and the lobe length measured before they were covered in soil again on each occasion in 1996. The nodules, which were used to measure nitrogenase activity, also had the lobe length measured. Three lobes per nodule were measured by a binocular

dissecting microscope, while green leaves and current shoots were collected to determine the N concentration on each occasion.

## 2.3 Acetylene reduction activity (ARA) of root nodules under field condition

$N_2$ -fixing activity was measured by the acetylene ( $C_2H_2$ ) reduction method (Hardy *et al.* 1968, 1973) on 8 occasions (14 days) in 1995 and 7 occasions (12 days) in 1996. The number of trees measured on each occasion was 1 to 5, the dbh of which ranged from 6 to 21 cm (Tobita *et al.* 2010). In 1996, one plant, which was suppressed by other trees, was contained within sample trees on each occasion, and known as a ‘suppressed tree’. The nodules, which ranged from 6 to 18 clusters per tree, were used for the measurements. Root nodules were excised with small segments of intact roots (> 2 cm long) and used to measure  $C_2H_2$  reduction activity (ARA). Since manipulations have to be minimized to avoid any reduction in nodule activity (Huss-Danell 1978; Wheeler *et al.* 1978), the  $C_2H_2$  reduction assays were conducted immediately after excision by incubation for 30 min with 10% acetylene in air within glass containers of 200 mL capacity. The containers were buried into soil at depth of 0.1 m beforehand and kept at soil temperature, so that observations were made under natural conditions as far as possible. On each occasion, the measurements were taken from 12:30 to 13:30, which was expected to show good activity (Sharma and Ambasht 1988; Sharma *et al.* 2010). After the incubation periods, gas samples from the container were transferred into pre-evacuated 3 mL draw Venoject tubes (Terumo Co., Tokyo, Japan) for gas chromatographic laboratory analysis. The production of ethylene was determined using a gas chromatograph equipped with a flame ionization detector (263-30, HITACHI, Tokyo, Japan). Hydrocarbon separation was achieved using a 1.0 m glass column packed with 100/120-mesh Porapak R. The detection temperature was maintained at 75°C, and the column temperature at 60°C.  $C_2H_2$  gas samples were also checked for potential ethylene contamination, while the  $C_2H_2$  reduction rates were corrected for the contamination level. A preliminary study under constant conditions showed that ethylene production was linear to the incubation time for at least 3 h. In fact, it is almost impossible to determine real nitrogenase activity values, because it varies according to many factors (Huss-Danell 1990). In this study, we used several assumptions to estimate nitrogenase activity and the  $N_2$  fixation rate of alder (Tjepkema *et al.* 1988). The first involved an actual conversion ratio of  $C_2H_2$  reduced to fixed  $N_2$  of 3:1. The  $C_2H_2$  reduction method has been found to underestimate  $N_2$  fixation in *Alnus* species (Schwintzer and Tjepkema 1997) and other species (Minchin *et al.* 1986).

During N<sub>2</sub> reduction but not during C<sub>2</sub>H<sub>2</sub> reduction, a variable part of the electron flow through nitrogenase is used to reduce H<sup>+</sup> to H<sub>2</sub>. On this basis, the ratio between N<sub>2</sub> and C<sub>2</sub>H<sub>2</sub> reduction would be expected to significantly exceed 3:1 (Winship and Tjepkema 1990). In fact, the actual ratio of acetylene reduction to N<sub>2</sub> fixation must be determined by using <sup>15</sup>N-labelled dinitrogen concurrently. Experimentally determined ratios between N<sub>2</sub> and C<sub>2</sub>H<sub>2</sub> reduction vary widely (Hardy and Havelka 1975), but those of 3.5–4.0 have been considered normal values (Schwintzer and Tjepkema 1997). In our study, we used the conversion rate 3:1 as a basis, and also calculated using a conversion rate of 4:1 for comparison to calculate the N<sub>2</sub> fixation from the ARA results in this alder stand. Another major difficulty of the C<sub>2</sub>H<sub>2</sub> reduction method is the C<sub>2</sub>H<sub>2</sub>-induced decline in nitrogenase activity (Layzell *et al.* 1984; Tjepkema *et al.* 1988; Schwintzer and Tjepkema 1997; Silvester *et al.* 2008). This is the phenomenon whereby when C<sub>2</sub>H<sub>2</sub> is added to the root nodules, nitrogenase activity declines within minutes. However, in *Alnus* spp., the error from using closed cuvettes for the C<sub>2</sub>H<sub>2</sub> reduction assay is probably small because the C<sub>2</sub>H<sub>2</sub>-induced decline is small (Schwintzer and Tjepkema 1994).

#### 2.4 Litterfall collection

Litterfall was collected by litter traps 0.25 m<sup>2</sup> in area and with a mesh size of 1 mm. Ten traps were systematically located in the plot and the litterfall was collected every month. Litterfall during winter was also collected by five litter traps of 2 × 2 m in area with a mesh size of 1 mm in 1996. Litterfall samples were air-dried and separated into individual organs of alder and other species, while frass was also weighed. Five grams of each fraction were oven-dried at 105°C and weighed to obtain the dry ratios.

We estimated the seasonal change in the attached leaf area in the alder canopy by utilizing data on shoot phenology and leaf litterfall as below, and these estimated values were known as corrected relative leaf area (*cr*-LA). First, the leaf area of leaves having emerged, attached, and fallen in the phenology data was represented as the relative ratio to the total emerged leaf area. Second, the cumulative leaf mass having fallen in the leaf litterfall data was also represented as the relative ratio to the total leaf litterfall. Two assumptions were made during this second calculation: (1) The seasonal changes in the mass of leaf litterfall were the same as those in the leaf area of leaf litterfall. (2) The leaf litterfall changed linearly between each occasion when we collected the litterfall. Third, the relative values of the fallen leaf area in phenology data were corrected to those of leaf litterfall. Finally, the relative values of the attached leaf area in phenology data were corrected as *cr*-LA using the corrected relative values of the fallen leaf area when the relative values of the emerged leaf area were fixed.

#### 2.5 Litterbag method

The decomposition rate of alder leaf litter was measured using a litter bag method. Newly fallen alder leaf litter was collected from the forest floor in November 1995 and immediately dried at 40°C. Three grams of air-dried alder leaves were then placed in each litter bag of 0.1 × 0.1 m in area with a mesh size of 1 mm. This weight per unit area of litter bag fell within the range of annual litterfall weights per unit area of forest floor. The litter bags were systematically set out in the study plot on 1 December 1995, and collected every month from May 1996. On each sampling occasion, 10 litter bags were collected. The constant (*k*) of leaf litter decomposition was calculated using the exponential decay model (Olson 1963; Tateno *et al.* 2007) as follows:

$$W_t/W_0 = \exp(-kt), \quad (2)$$

where *W*<sub>0</sub> is the original litter weight, and *W*<sub>*t*</sub> is the litter weight after a given period of time *t*.

#### 2.6 Forest floor and mineral soil

The vegetation of the forest floor and organic matter of the A<sub>0</sub> layer were collected from three 0.5 × 0.5 m quadrates every month. Samples of the A<sub>0</sub> layer were separated into twigs, cones, coarse organic matter (≥2 mm) and fine organic matter (<2 mm). In August 1996, the vertical distribution of carbon and N in the mineral soils was measured at the center part of this study site. Soil samples were collected using 400 ml soil core every 0.05 m of the soil surface to 0.3 m and subsequent depth of 0.4, 0.6, and 0.85 m. Fine soils were used for chemical analysis and the air-dried weight of each fraction was measured. Five grams of each fraction were oven-dried at 105°C and weighed to obtain a dry weight ratio.

#### 2.7 Chemical analysis

The nitrogen and carbon content of litterfall, green leaves, initial and decomposing leaves of the litterbag method, herbaceous plants of the forest floor, organic matter of the A<sub>0</sub>-layer, and fine soil were analyzed by automatic gas chromatography (MT-600; Yanaco, Kyoto, Japan).

#### 2.8 Data analysis

Seasonal variation in acetylene reduction activity (ARA) per unit nodule mass was analyzed using a general linear model (R Ver 2.6.1., R Development Core Team 2008). The statistical model used allows correlated variables, year, tree size (dbh), corrected relative leaf area (*cr*-LA), nodule size as

presented by the root diameter at nodule base, soil temperature at a soil depth of 0.1 m and the distance from the stem, to be analyzed. The three factors (year, tree size, and distance from the stem) were then eliminated from the model by step-AIC procedure. Finally, the nodule size and *cr*-LA were selected to explain the variations in ARA (AIC = 3082, Residual deviance = 467.7, Null deviation = 698.2). Another model which added soil temperature to the above dual factor model showed the result as AIC = 3082.8, Residual deviance = 466.72. There were only minor differences between the two models, while in this study, the following model, which used three variables, *cr*-LA, nodule size, and soil temperature, was adopted for the ARA prediction:

$$[\text{ARA}] = \exp(1.12 + 0.033 [\text{cr-LA}] - 0.247[\text{nodule size}] + 0.036[\text{soil temperature}]), \quad (3)$$

As a comparison, we also calculated using the first model (equation 4), which has two variables, *cr*-LA and nodule size, and using the other model with only soil temperature (equation 5).

$$[\text{ARA}] = \exp(1.44 + 0.037[\text{cr-LA}] - 0.248[\text{nodule size}]), \quad (4)$$

$$[\text{ARA}] = \exp(-7.61) \times [\text{soil temperature}]^{3.83} \quad (5)$$

In this alder stand, the nodule size class structure per tree depended on the tree size (dbh), and was described by Weibull distribution (Tobita *et al.* 2010) as follows:

$$f(x) = 100(c/b)(x/b)^{c-1} \exp\{-f(x/b)^c\},$$

$$[b] = 0.044[\text{dbh}] + 1.84, [c] = 0.045[\text{dbh}] + 1.36 \quad (6)$$

The relationship between nodule size and each nodule biomass was calculated using nodule data, which showed no symptoms of decay.

$$[\text{Nodule biomass}] = 0.0413[\text{nodule size}]^{1.8498},$$

$$R^2 = 0.6477, \quad n = 399 \quad (7)$$

Using this equation, the average nodule mass of each size class was calculated. In this estimation, we assumed that these nodules showed no decay. We constructed a hypothetical structure of nodules with 1 g mass per tree using equations (6) and (7), where upon the mass of each nodule size class was calculated per tree, and termed 'the nodule mass ratio'. The annual C<sub>2</sub>H<sub>2</sub> reduction was then calculated for each size class of nodule using equation (3) and multiplied by the calculated nodule mass ratio. The sum of the calculated values of all nodule size classes showed the annual C<sub>2</sub>H<sub>2</sub> reduction in the hypothetical structure of nodules with 1 g mass per tree. The nodule biomass per tree was estimated

using the equation of the relationship between tree size and nodule biomass (Tobita *et al.* 2010) as below.

$$[\text{Nodule biomass}] = 0.442[\text{dbh}]^{2.01} \quad (8)$$

The estimated nodule biomass per tree was multiplied by the calculated annual C<sub>2</sub>H<sub>2</sub> reduction by the hypothetical structure of nodules with 1 g mass per tree, the results of which showed the annual C<sub>2</sub>H<sub>2</sub> reduction per tree. As above-mentioned, both the conversion ratio of C<sub>2</sub>H<sub>2</sub> reduced to N<sub>2</sub> fixed, 3:1 and 4:1, were used. In this alder stand, we calculated the N<sub>2</sub> fixation as the sum of N<sub>2</sub> fixation of all trees divided by the plot area (0.105 ha). In addition, the amount of N<sub>2</sub> fixation was also calculated as the sum of N<sub>2</sub> fixation of all trees divided by the sum of the target area within 1 m around the stems of this study plot (0.0337 ha) as comparison. Analysis of variance was used to compare ARA between tree size (dbh) on each occasion. The statistical significance of differences between tree size (dbh) was tested with the Turkey-Kramer *post hoc* test at a probability level  $P < 0.05$ .

### 3. Results

#### 3.1 Phenology

The time taken to reach the maximum growth rates of shoot length, leaf area, stem diameter, nodule length, and catkin length of alder (*Alnus hirsuta* var. *sibirica*) were observed in this order (figure 1). In early May, the first to third leaves unfolded but did not fully expand (figure 1A). In June, at least three leaves emerged in almost all shoots measured, while about 60% of the first leaves and 30% of the second leaves had already fallen (figure 1A). Some shoots produced 14 leaves by August. In early August, the emergence of new leaves stopped, while the attached leaf area per shoot peaked and remained unchanged to early September. The number of fallen leaves soared in early October. In early November, all leaves defoliated on the lower part of the crown, for which we measured shoot phenology, but a few leaves remained attached on the upper part of crown in the same tree.

The elongation of current shoot started at almost the same time as the expansion of leaves, and stopped in the mid-August (figure 1B), while the stem diameter at breast height (dbh) increased measurably from early June to early September (figure 1C) and the length of the nodule lobes increased measurably from August to September (figure 1D). Reproductive organs (male and female catkins) elongated from August until late September (figure 1E).

The seasonal changes in the increment in dbh showed similar patterns over the 2 years, but the increment in dbh showed significant differences over the same period, and among tree sizes (figure 2). The mean increments of dbh ( $\pm$ SE) in 1995 and 1996 were 0.54 ( $\pm$ 0.03) cm and 0.45

( $\pm 0.03$ ) cm, which corresponded to a mean increment ratio per year ( $\pm$ SE), of 3.96 ( $\pm 0.20$ )% in 1995 and 3.07 ( $\pm 0.17$ )% in 1996, respectively. The relative increment of dbh in relatively small trees in this alder stand, which were suppressed by the crowns of neighbors, was smaller than those of the bigger ones.

### 3.2 Litterfall

Litterfall data showed that leaves began to fall in June and increased through growth periods, one peak which emerged in November (figure 3). The litterfall during the growth periods in 1995 was 3.77 ton ha<sup>-1</sup> year<sup>-1</sup> (3.87 ton ha<sup>-1</sup> year<sup>-1</sup> in 1996) (table 1), while the leaf litter of alder was 3.07 ton ha<sup>-1</sup> year<sup>-1</sup> (3.10 ton ha<sup>-1</sup> year<sup>-1</sup> in 1996) and occupied 81.4% (80.0% in 1996) of total litterfall in 1995. The insect feces were 105 kg ha<sup>-1</sup> year<sup>-1</sup> (89 kg ha<sup>-1</sup> year<sup>-1</sup> in 1996). The litterfall during the winter season in 1996 was 238 kg ha<sup>-1</sup> year<sup>-1</sup>, which comprised branches (63.7%) and reproductive organs (almost all male flowers) (34.2%). The yearly litterfall included in the winter season in 1996 was 4.11 ton ha<sup>-1</sup> year<sup>-1</sup>, 5.8 % of which was the litterfall during winter.

### 3.3 Seasonal variations in acetylene reduction activity (ARA) of root nodules

Although the soil temperature at a soil depth of 0.1 m was about 8°C in late April, no C<sub>2</sub>H<sub>2</sub> reduction activity (ARA) was detected before leaf emergence (figure 4). ARA appeared in early May after the leaves unfolded and peaked in early August (68.0  $\mu$ mol C<sub>2</sub>H<sub>4</sub> g<sup>-1</sup> nodule h<sup>-1</sup> in 1995; 28  $\mu$ mol g<sup>-1</sup> C<sub>2</sub>H<sub>4</sub> nodule h<sup>-1</sup> in 1996) when both leaf area and soil temperature peaked during the growth periods. In early November 1995, the several leaves remained in the crown and low but significant ARA was detected, though the soil temperature was about 5°C under snow and lower than that in late April. ARA was no longer measurable in early December after all the leaves had fallen. Though ARA varied significantly in early August over the 2 years, the seasonal changes showed no significant differences over the 2 years (figure 4), nor was there any consistent correlation between tree size (dbh) and ARA on each occasion (figure 5). The suppressed trees did not always show lower ARA compared to those of larger size.

### 3.4 The estimation of N<sub>2</sub> fixation

In this study, a generalized linear model, which used three variables (equation (3)), corrected relative leaf area (*cr*-LA), nodule size as presented by the root diameter at nodule base, and soil temperature at a soil depth of 0.1 m, was adopted to predict ARA. ARA increased in accordance with *cr*-LA and

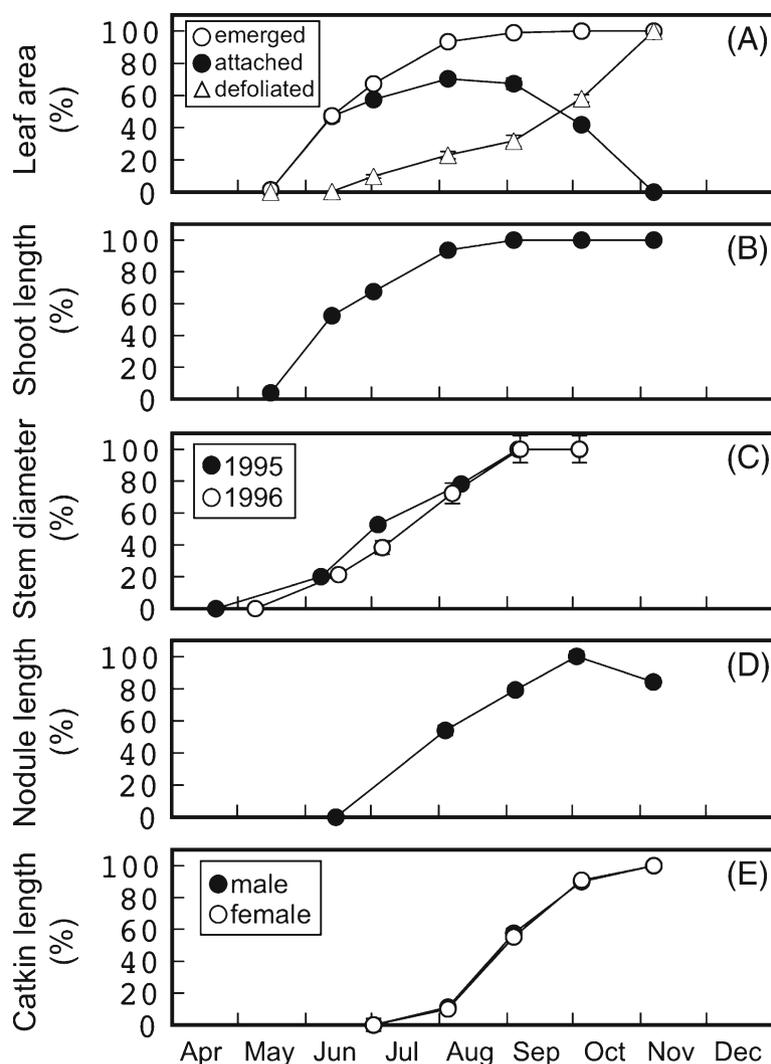
soil temperature. Nodule size adversely affected ARA, which decreased with increasing nodule size. The mean (min-max) amount of ethylene (C<sub>2</sub>H<sub>4</sub>) produced by unit nodule mass in a year was 0.071 (0.069–0.072) mol C<sub>2</sub>H<sub>4</sub> g<sup>-1</sup> nodule year<sup>-1</sup> in 1995 and 0.072 (0.069–0.073) mol C<sub>2</sub>H<sub>4</sub> g<sup>-1</sup> nodule year<sup>-1</sup> in 1996. When a theoretical conversion ratio of 3 was used for these ARA methods, the annual N<sub>2</sub> fixation of alder by unit nodule mass was estimated at 0.66 (0.64–0.67) gN g<sup>-1</sup> nodule year<sup>-1</sup> in 1995 (0.67 (0.65–0.68) gN g<sup>-1</sup> nodule year<sup>-1</sup> in 1996) (table 2). If we used a molar ratio of 4, the annual N<sub>2</sub> fixation was estimated at 0.50 (0.48–0.50) gN g<sup>-1</sup> nodule year<sup>-1</sup> in 1995 and 0.50 (0.49–0.51) gN g<sup>-1</sup> nodule year<sup>-1</sup> in 1996.

In this stand, the average (min-max) nodule biomass per tree within 1 m of the outer edge of stems in April 1995 was estimated at 77.5 g tree<sup>-1</sup> (5.6 to 199.2 g tree<sup>-1</sup>) using the relationships between tree size (dbh) and nodule biomass per tree (equation (7); Tobita *et al.* 2010). When we assumed that this relationship could be applied in 1996, the average (min-max) nodule biomass per tree within 1 m of the outer edge of stems in May 1996 was estimated at 88.3 g tree<sup>-1</sup> (6.5 to 227.2 g tree<sup>-1</sup>). From these results, when we used a theoretical conversion ratio of 3, the average (min-max) N<sub>2</sub> fixation per tree were estimated to be 50.6 (3.7 to 126.6) gN tree<sup>-1</sup> in April 1995, and 58.5 (4.3 to 145.5) gN tree<sup>-1</sup> in May 1996, respectively. The results of summing up the calculated N<sub>2</sub> fixation per tree in this alder stand and dividing by the plot area (0.105 ha) showed an estimated amount of N<sub>2</sub> fixation per area of 56.4 kgN ha<sup>-1</sup> year<sup>-1</sup> (60.2 kgN ha<sup>-1</sup> year<sup>-1</sup> in 1996), and 42.3 kgN ha<sup>-1</sup> year<sup>-1</sup> (45.2 kgN ha<sup>-1</sup> year<sup>-1</sup> in 1996) when we used a theoretical molar ratio of 4.

To compare the estimated values, we conducted the same calculation procedure of N<sub>2</sub> fixation in 1995 using two other models (equations (4) and (5)). In model 2 (equation (4)), ARA was represented by two factors, *cr*-LA and nodule size, while the amount of N<sub>2</sub> fixation was estimated at 55.6 kgN ha<sup>-1</sup> year<sup>-1</sup>. Conversely, in model 3 (equation (5)), ARA was represented by soil temperature alone, and the amount of N<sub>2</sub> fixation was calculated at 68.0 kgN ha<sup>-1</sup> year<sup>-1</sup>. Though estimation by model 2 showed only minor differences from the result of model 1, estimation by model 3 showed a 20% increase compared to the result of model 1.

### 3.5 Nitrogen content in litterfall

The nitrogen (N) content of green leaves at the upper crown of the alder peaked just after expansion (4.01  $\pm$  0.12%), was 3.29  $\pm$  0.15% in August, and decreased with increasing age from September (data not shown). There were no significant differences in leaf N content between the lower canopy (3.50  $\pm$  0.07% in August) and upper canopy. In November, the difference in N content between green leaves on the upper canopy layer (2.78  $\pm$  0.06%) and fallen leaves (2.56  $\pm$  0.03%)



**Figure 1.** Phenology of *Alnus hirsuta* var. *sibirica*: leaf area per current shoot (leaf area of emerged leaves (open circles), attached leaves (closed circles), and defoliated leaves (open triangles)) (A), current shoot length (B), stem diameter growth at breast height (dbh) in 1995 (closed circles) and 1996 (open circles) (C), length of nodule lobe (D), and length of catkins for male (closed circles) and female (open circles) (E). Values shown are ratios to the maximum of means  $\pm$  SE (A, B:  $n = 93$ , C:  $n = 50$ , D:  $n = 156$ , E:  $n = 76$  to 102).

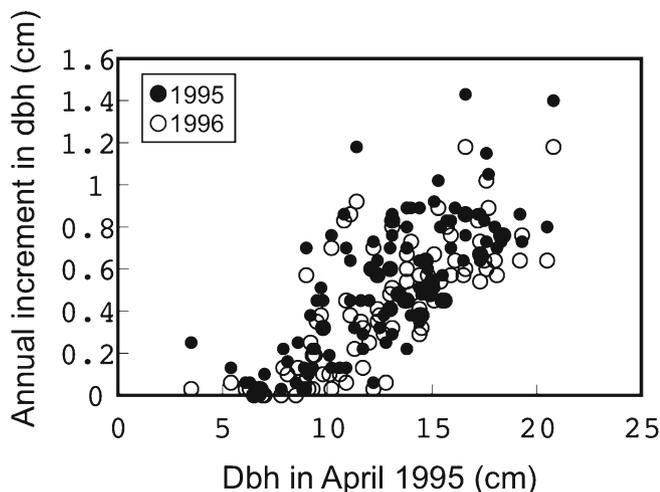
corresponded to 7.9% of those of green leaves. When the N content of green leaves in August was compared to those of fallen leaves, the pre-senescence depletion in N was 22.2% of those of green leaves.

The average N content of litter during the growth period was 2.75% in leaves, 2.15% in stipules, 1.37% in shoots and buds and 1.89% in reproductive organs (table 3). The total N input ( $\pm$ SE) by litterfall, except for the winter season, was  $94.9 (\pm 3.56) \text{ kg ha}^{-1} \text{ year}^{-1}$  in 1995 and  $94.8 (\pm 4.02) \text{ kg ha}^{-1} \text{ year}^{-1}$  in 1996. The N content in leaf litter of alder was  $84.9 (\pm 2.11) \text{ kg ha}^{-1} \text{ year}^{-1}$  in 1995 and  $84.3 (\pm 2.49) \text{ kg ha}^{-1} \text{ year}^{-1}$  in 1996, which was equivalent to N content of 89.4 and 89.3% in whole litterfall except for the winter season

each year. The N content of the litterfall during the winter season was  $3.62 \text{ kg ha}^{-1} \text{ year}^{-1}$ , which was 3.66% of the N content in the whole litterfall including winter, while the N content in leaf litter of alder occupied 85.7% of the N content in the whole litterfall.

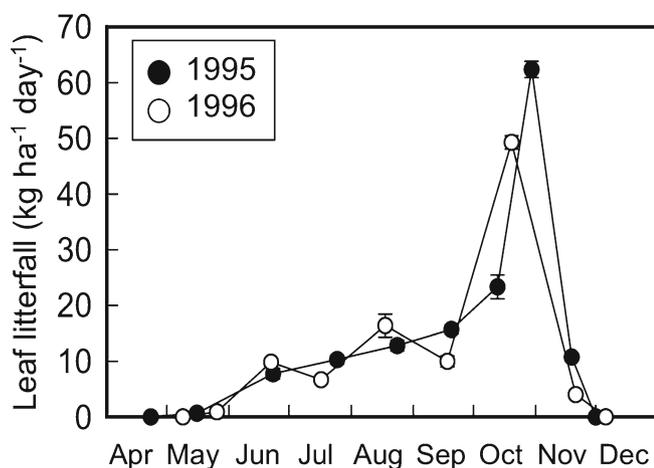
### 3.6 Decomposition of leaf litter

The weight of leaf litter of alder within the litterbag decreased to 40% of the initial value after five months in winter (figure 6A), while the rate of weight loss in leaf litter increased during summer (from July to September) and



**Figure 2.** The annual increment in the stem diameter (dbh) of *Alnus hirsuta* var. *sibirica* in 1995 (closed circles) and 1996 (open circles).

decreased thereafter. The weight loss in a year was 70% from the initial mass, while the coefficient  $k$  in the exponential function (equation (2)) was  $1.13 \text{ year}^{-1}$ . The loss in N content of the leaf litter during winter was 25% from the initial value and smaller than the mass loss (figure 6A). Subsequently, the N content of leaf litter decreased at an equivalent or faster rate compared to the mass. The C/N ratio of the leaf litter of alder was lower than 20 during the early decomposition stage (figure 6B). The C/N ratio decreased to 12 during the initial six months and remained unchanged thereafter, while the N content decreased by 58.4% in a year.



**Figure 3.** Seasonal variations in leaf litterfall caught by the litter traps in the *Alnus hirsuta* var. *sibirica* stand in 1995 (closed circles) and 1996 (open circles).

Consequently,  $45.8 \text{ kg ha}^{-1} \text{ year}^{-1}$  N was supplied into soil in a year by the decomposition of leaf litter.

### 3.7 Forest floor

The biomass of herbaceous plants on the floor of this alder stand peaked in August ( $2.4 \pm 0.76 \text{ ton ha}^{-1}$ ), while  $A_0$  layer on soil showed the smallest value in August. In August, the total amount of organic matter on forest floor (herbaceous species +  $A_0$  layer) was  $9.3 \text{ ton ha}^{-1}$ . Within the  $A_0$  layer, the organic matter  $> 2 \text{ mm}$  was 49.9%, the organic matter  $< 2 \text{ mm}$  was 17.9%, twigs were 31.6%, and fruit were 0.6%, respectively. The N content in herbaceous biomass and the  $A_0$  layer was  $57.9 \text{ kg ha}^{-1}$  and  $146.2 \text{ kg ha}^{-1}$ , respectively.

### 3.8 Soil N fractions

Carbon (C) and N concentration in the mineral soil of this alder stand peaked at a depth of 0–0.05 m (C: 4.9%, N: 0.4%), and decreased sharply at 0.05–0.10 m (C: 1.1%, N: 0.09%). At deeper soil layers, C and N concentrations were less than one-tenth of the surface soil (C:  $< 0.6\%$ , N:  $< 0.05\%$ ), while the accumulation of C and N at a depth of 0–1.0 m in the mineral soil were 32.0 and 3.4  $\text{ton ha}^{-1}$ , respectively. About 30% of all N in the mineral soil accumulated in the surface soil (0–0.05 m depth). The N accumulation in the mineral soil when this site was degraded by road construction was estimated at  $2.0 \text{ ton ha}^{-1}$ , when the initial N concentration was assumed to be the same as those at a depth of 0.30 m in mineral soil (0.026%). Total N accumulation after disturbance for 21 years (1975–1996) was estimated at  $1.4 \text{ ton ha}^{-1}$ , while the averaged N accumulation rate in a year was estimated at  $66.7 \text{ kg ha}^{-1} \text{ year}^{-1}$ . This value was simply calculated as the total N having accumulated in the mineral soil divided by the number of years since the road construction.

## 4. Discussion

### 4.1 The relationship between phenology and ARA of *Alnus hirsuta* var. *sibirica*

No consistent correlation was found between the acetylene reduction activity per unit nodule mass (ARA) and tree size (dbh) in this alder (*Alnus hirsuta* var. *sibirica*) stand (figure 5). Suppressed trees also showed the same degree of ARA as the crown trees through the growth period, though the increment of dbh in suppressed trees was less than those of crown trees (figure 2). Nodule biomass per tree, however, increased with tree size (Tobita et al. 2010). These results indicate that the amount of  $\text{N}_2$  fixation per tree was lower in suppressed trees than those of

**Table 1.** The litterfall in *Alnus hirsuta* var. *sibirica* stand in 1995 (21 April to 1 December) and in 1996 (9 May to 6 December)

Litter component	1995 (21.Apr - 1.Dec)		1996 (9.May - 6.Dec)		6.Dec 1996 - 9. May 1997 (kg ha <sup>-1</sup> )
	(kg ha <sup>-1</sup> )	(%)	(kg ha <sup>-1</sup> )	(%)	
Leaves (alder)	3065	(81.4)	3097	(80.0)	-
Branches	246	(6.5)	458	(11.8)	152
Stipules	121	(3.2)	111	(2.9)	-
Flowers	101	(2.7)	42	(1.1)	81
Leaves (other spp.)	118	(3.1)	59	(1.5)	0
Insect feces	105	(2.8)	89	(2.3)	0
Others	11	(0.3)	14	(0.4)	5
Total	3767	(100)	3870	(100)	238

In 1996, the litterfall during winter (6 December to 9 May 1997) was also collected. Values in parentheses show the percentage of each component to total litterfall within each year.

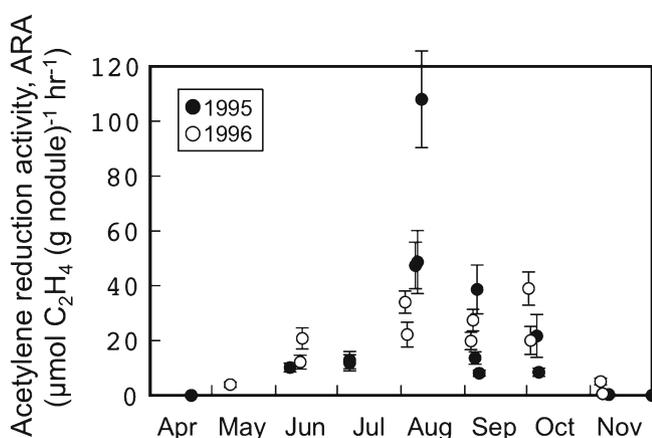
crown trees because of the difference in nodule biomass per tree, not the decrease in nodule activity.

The interaction of several biotic and abiotic factors will affect seasonal variations in the nitrogenase activity of actinorhizal plants (Schwintzer *et al.* 1982). The seasonal pattern of ARA in alder observed here and its relationship with shoot phenology was typical of winter-deciduous actinorhizal plants, and similar patterns of ARA under field conditions have also been observed in various other winter-deciduous actinorhizal plants including *Alnus* species, *Hippophae* and *Myrica* (Schwintzer *et al.* 1982; Sharma *et al.* 2010).

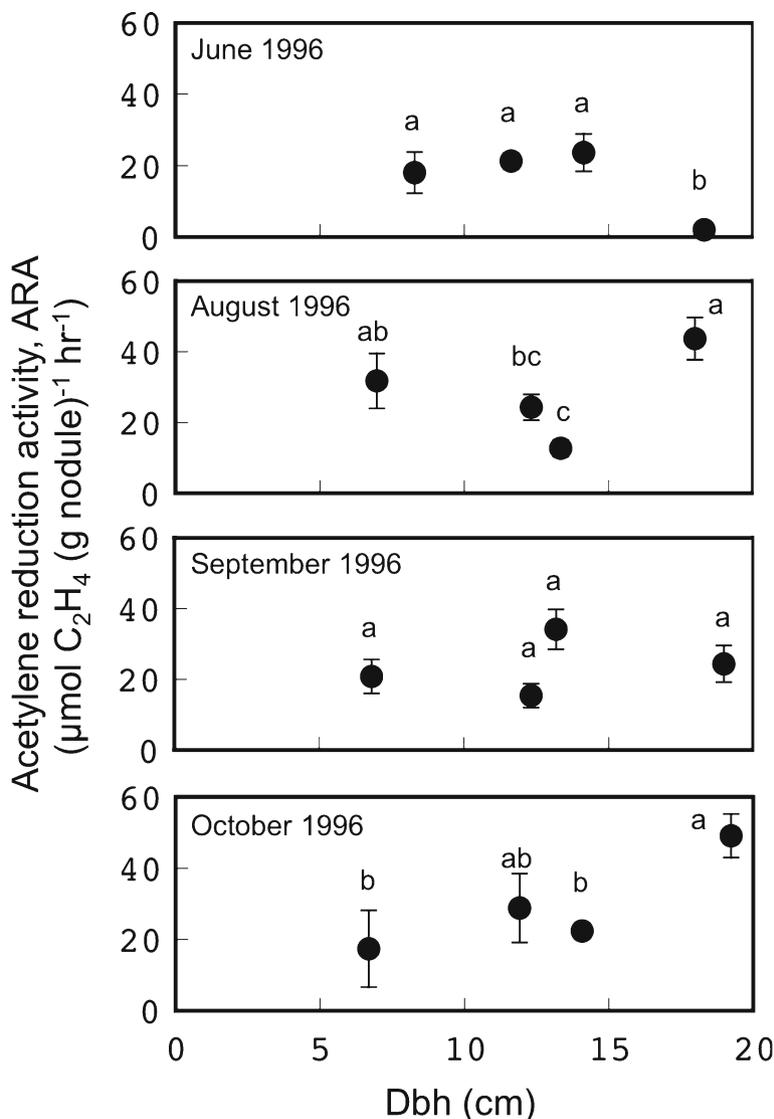
In this alder stand, there were large variations in ARA in summer over a year and 2 years respectively. One possible

reason was the difference in the amount of reproduction over 2 years. The large increment in the mass of female cones started earlier than the female and male flowers in the *Alnus* stand (Pizelle 1984; Hasegawa and Takeda 2001). In addition, in 1996, levels of female and male flower production exceeded those in 1995 (Hasegawa and Takeda 2001). It was assumed that a larger investment of energy in reproduction was associated with a decreased allocation of photosynthate to stem growth. Pizelle (1984) also reported that summer nitrogenase activity was low when the development of fruit was rapid but increased when the maturation of seeds was complete in 20- to 30-year-old trees of *Alnus glutinosa*. Similarly, the proportion of photosynthate allocated to root nodules was assumed to be limited in August when the growth rate of reproductive tissues was fast. Such variations in nitrogenase activity might be explained by competition between nodules and developing fruit for assimilates.

Another potential explanation for the large variations in ARA during summer may be the difference in weather conditions (Huss-Danell 1990). In August 1995, ARA was measured on three different days. On the third day, it rained for the first time in 20 days before measurement, and the ARA reading exceeded those of the other two days. Previous studies showed ARA decreased in response to drought stress (Hawkins and MacDonald 1994; Sharma *et al.* 2010), and also showed that ARA of nodules in *Alnus nepalensis* and *Alnus tenuifolia* was high during the rainy season (Sharma and Ambasht 1988; Sharma *et al.* 2002; Uliassi and Ruess 2002). In addition, Huss-Danell *et al.* (1992) indicated that ARA might be affected by both the environmental conditions of the day and by the day. It was assumed that the drought and rain around the day of measurements date affected the significant variations in ARA in summer. The difference in the monthly amount of precipitation in summer might also affect the large variation of ARA in summer between 2 years in this stand.



**Figure 4.** Seasonal change in nitrogen fixing activity (acetylene reduction activity; ARA) in excised nodules of *Alnus hirsuta* var. *sibirica* under field condition in 1995 (closed circle) and 1996 (open circle). Values shown are means  $\pm$  SE ( $n = 6$  to 18 in 1995,  $n = 6$  to 20 in 1996). No ARA was detected in April and December in 1995.



**Figure 5.** The relationships between the stem diameter (dbh) of *Alnus hirsuta* var. *sibirica* and acetylene reduction activity (ARA) of their nodules from June to October in 1996. Values shown are means  $\pm$  SE ( $n = 9-10$ ). The smallest tree among the sample trees each month was the suppressed tree in 1996. Different letters indicate statistically significant differences between species at  $P < 0.05$  (Tukey–Kramer test).

#### 4.2 Contribution of N<sub>2</sub> fixation to N cycle in this *Alnus hirsuta* var. *sibirica* stand

In this alder stand, the nodule biomass and size class distribution of nodules as presented by the root diameter at nodule base per tree were estimated by tree size in dbh (Tobita *et al.* 2010). Utilizing these relationships and the results of this study, we could calculate the N<sub>2</sub> fixation rate of all trees in this plot. In naturally regenerated stands, the distribution of trees may not be as orderly as those of managed forests (Tobita *et al.* 2010). In such naturally regenerated stands, it may be difficult to estimate N<sub>2</sub> fixation from area-based data, and it might be useful to calculate it from individual-based data.

In the model for predicting ARA, nodule size as represented by the root diameter at nodule base adversely affected ARA. This result consists that larger nodules have lower specific nitrogenase activity because they have a higher ratio of woody tissue to symbiotically active tissue, which occurs only at the tip of nodule lobes (Sharma and Ambasht 1984; Huss-Danell 1990; Hurd *et al.* 2001).

The estimated annual N<sub>2</sub> fixation rate in this stand, 56.4 kgN ha<sup>-1</sup> year<sup>-1</sup>, appeared reasonable but relatively low in comparison with the ranges, several kgN ha<sup>-1</sup> year<sup>-1</sup> to 320 kgN ha<sup>-1</sup> year<sup>-1</sup>, previously reported (Hibbs and Cromack 1990; Huss-Danell 1990; Hurd and Schwintzer 1996; Hurd *et al.* 2001; Sanboen *et al.* 2002; Sharma *et al.* 2002; Lee and

**Table 2.** The results of estimation of N<sub>2</sub> fixation using three models, models 1, 2, and 3

		Model 1 ( <i>cr</i> -LA, nodule size, soil temperature)	Model 2 ( <i>cr</i> -LA, nodule size)	Model 3 (soil temperature)
Conversion ratio 3 : 1				
N <sub>2</sub> fixation per nodule mass	(gN g <sup>-1</sup> nodule yr <sup>-1</sup> )	0.66 (0.64 - 0.67)	0.65 (0.63 - 0.66)	0.80
N <sub>2</sub> fixation per tree	(gN tree <sup>-1</sup> yr <sup>-1</sup> )	50.6 (3.7 - 126.6)	49.9 (3.7 - 124.7)	61.0 (4.4 - 156.5)
N <sub>2</sub> fixation per all trees	(kgN all trees <sup>-1</sup> yr <sup>-1</sup> )	5.9	5.8	7.1
N <sub>2</sub> fixation per area	(kgN ha <sup>-1</sup> yr <sup>-1</sup> )	56.4	55.6	68.0
Conversion ratio 4 : 1				
N <sub>2</sub> fixation per nodule mass	(gN g <sup>-1</sup> nodule yr <sup>-1</sup> )	0.50 (0.48 - 0.50)	0.49 (0.48 - 0.50)	0.60
N <sub>2</sub> fixation per tree	(gN tree <sup>-1</sup> yr <sup>-1</sup> )	38.0 (2.8 - 95.0)	37.4 (2.7 - 93.5)	45.7 (3.3 - 117.4)
N <sub>2</sub> fixation per all trees	(kgN all trees <sup>-1</sup> yr <sup>-1</sup> )	4.4	4.4	6.9
N <sub>2</sub> fixation per are	(kgN ha <sup>-1</sup> yr <sup>-1</sup> )	42.3	41.7	65.4

N<sub>2</sub> fixation per unit nodule mass (gN g<sup>-1</sup> nodule year<sup>-1</sup>), N<sub>2</sub> fixation per tree (gN tree<sup>-1</sup> year<sup>-1</sup>), N<sub>2</sub> fixation per all trees in this study plot (kgN all trees<sup>-1</sup> year<sup>-1</sup>), and N<sub>2</sub> fixation per area (kgN ha<sup>-1</sup> year<sup>-1</sup>) were shown for both conversion rates 3:1 and 4:1. Values in parentheses show the minimum and maximum values. We used a study plot area of 0.105 ha and stand density of 1114 ha<sup>-1</sup> for the calculation. The nodule biomass per tree was calculated using the equation (Tobita *et al.* 2010), [nodule biomass] = 0.442 [dbh]<sup>2.01</sup>.

Son 2005; Son *et al.* 2007). However, except the two highest values over 300 kgN ha<sup>-1</sup> year<sup>-1</sup> (Zavitkovski and Newton 1968; Newton *et al.* 1968), which measured in *Alnus rubra* stand at western Oregon, the previous reported ranges were up to 170 kgN ha<sup>-1</sup> year<sup>-1</sup>. *Alnus hirsuta* var. *sibirica* with a pronounced and lengthy winter season would not be expected to have among the highest estimated stand N<sub>2</sub> fixation rates.

When the N<sub>2</sub> fixation is based on stand density as above-mentioned, there may be an underestimation because the total area within 1 m of the outer edge of stems (0.0337 ha) covers only about one-third of this plot area (0.105 ha). If we calculate using this covered area of 0.0337 ha, the annual N<sub>2</sub> fixation rate in this stand will be estimated at 175.85 kgN ha<sup>-1</sup> year<sup>-1</sup> in 1995, with conversion ratio 3. This value also appeared reasonable but relatively high in comparison with the ranges previously reported. Conversely, in this study, since the estimation of nodule biomass was conducted for a

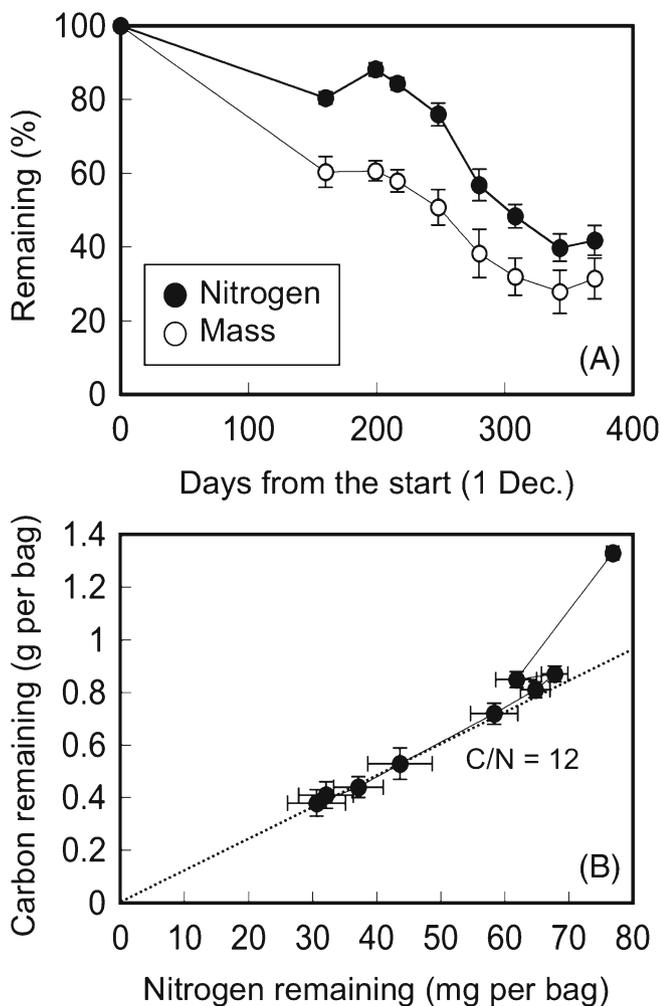
limited area as above-mentioned (Tobita *et al.* 2010), the calculations of nodule biomass per individual for larger trees would be underestimated. Accordingly, these procedures to estimate the N<sub>2</sub> fixation rate may produce underestimation.

We will discuss the contribution of N<sub>2</sub> fixation to the N cycle in this stand using the results of N<sub>2</sub> fixation based on stand density and using conversion ratio 3, 56.4 kgN ha<sup>-1</sup> year<sup>-1</sup>. This calculated N<sub>2</sub> fixation rate per year corresponded to annual N content of leaf litter in this stand of 66.4% (figure 7). This N<sub>2</sub> fixation value was also equivalent to 123.2% of the N supplied to the soil by the decomposition of fresh leaf litter in a year. The data was from different years, but interestingly, the estimated N<sub>2</sub> fixation rate remained almost the same as the maximum N contents in herbaceous plants in August (104.0%), and was also equal to 90.3% of the calculated average N accumulation rate into the mineral soil.

**Table 3.** Nitrogen content of litterfall at this *Alnus hirsuta* var. *sibirica* stand in 1995 (21 April to 1 December) and 1996 (9 May to 6 December).

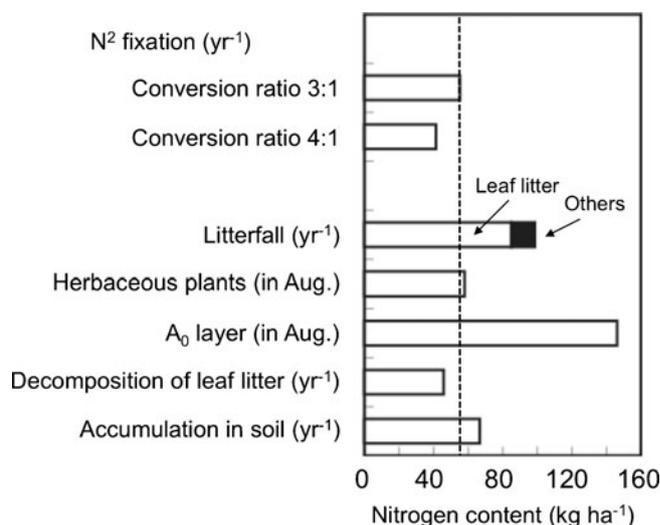
Litter component	1995 (21.Apr - 1.Dec)		1996 (9.May - 6.Dec)		N content (%)
	(kg ha <sup>-1</sup> )	(%)	(kg ha <sup>-1</sup> )	(%)	
Leaves (alder)	84.9	(89.4)	84.3	(88.9)	2.75
Branches	3.4	(3.5)	6.2	(6.6)	1.37
Stipules	2.6	(2.7)	2.4	(2.5)	2.15
Flowers	1.9	(2.0)	0.8	(0.8)	1.89
Leaves (other spp.)	2.1	(2.3)	1.1	(1.1)	1.83
Total	94.9	(100)	94.8	(100)	

Values in parentheses show the percentage of each component to total litterfall within each year. The value of the nitrogen (N) content of each component was shown as the mean values.



**Figure 6.** The remaining mass (open circles) and nitrogen (closed circles) during decomposition in leaf litter of *Alnus hirsuta* var. *sibirica* (A). The initial value was shown as 100%. The relationship between N and C in the leaf litter during decomposition (B). The dashed line showed the C/N = 12. Values shown are means  $\pm$  SE ( $n = 10$ ).

Sharma and Ambasht (1988) reported that the amount of  $N_2$  fixation in *Alnus nepalensis* plantations reduced from 117.29 to 29.05  $kg\ ha^{-1}\ year^{-1}$ , while  $N_2$  fixation as a percentage of total N uptake also declined from 33 to 11% with increasing stand age from 7- to 56-year-old. Although we did not measure the amount of N absorbed in this alder stand,  $N_2$  fixation occupied 66.4% of the N content in litterfall in this stand as above-mentioned (figure 7). It is also reported that the percentage of N content returned by litterfall of the total N uptake was about 35, 54, 57, 59% and 56% respectively in 7-, 17-, 30-, 46- and 56-year-old *Alnus nepalensis* plantations (Sharma and Ambasht 1988; Sharma 1993). If we use these reported values (35–59%) for the percentage N content returned by litterfall of the total N uptake in this stand, the N



**Figure 7.** Nitrogen flow and stock in this *Alnus hirsuta* var. *sibirica* stand. The estimated  $N_2$  fixation rate was shown using both 3:1 and 4:1 conversion ratio. For the data of litterfall, the white part showed leaf litter, while the black part showed other kinds of litter.

uptake rate will be calculated within the range 166.8 to 281.2  $kgN\ ha^{-1}\ year^{-1}$ , while the percentage contribution of  $N_2$  fixation of total N uptake in this stand will be calculated as 19.6 to 33.1%. Other actinorhizal plant, *Myrica faya*, showed that the nodule biomass could not increase with enhancing tree size, when the contribution rate of  $N_2$  fixation to absorbed N decreased due to increasing N content in soil (Vitousek and Walker 1989). Increased nitrate in soils can inhibit nodulation and reduce  $N_2$  fixation (Wall and Berry 2008). In our study, the nodule biomass of individual trees increased with tree size (dbh), and an allometrical relationship remained between them (Tobita et al. 2010). Therefore, these results suggested that  $N_2$  fixation still contributed to the significant portion of N economy in this alder stand.

#### 4.3 N accumulation in the soil of *Alnus hirsuta* var. *sibirica* stand

Nutrient resorption from leaves before senescence in autumn will be an important strategy to acclimatize to infertile soil conditions due to decrease the loss of nutrient (Chapin 1980) and increase the nutrient use efficiency (Vitousek 1982; Enoki et al. 1997). The N resorption rate per unit leaf mass of non- $N_2$ -fixing deciduous tree species has been reported at 33 to 66% (Kramer and Kozłowski 1979). In this alder stand, the N resorption rate per unit leaf mass was calculated as 22.2% when we compared it with the N content of mature leaves in August. Low N resorption in autumn was also

reported for *Alnus firma* (Tateno 2003) and *Alnus glutinosa* (Dawson and Funk 1981). It is proposed that *Alnus* species could maintain high N content in green leaves and relatively high photosynthetic rate in autumn season due to lower N resorption than other non-N<sub>2</sub>-fixing deciduous broadleaved species (Tateno 2003). It was also suggested that these properties (low N resorption rate) might help retain relatively high N<sub>2</sub> fixation activity in autumn by supplying sufficient photosynthate to the nodules (figure 4).

One of the features of *Alnus* species is the high susceptibility of the leaves to herbivores (Kikuzawa *et al.* 1979; Tadaki *et al.* 1987). Cyr and Pace (1993) showed that arthropod herbivores generally consumed 10% to 20% of the aboveground net primary production. Herbivorous insects flux considerable amounts of N from the forest canopy to the soil in the form of frass (Meehan and Lindroth 2007). Consequently, the Frass-derived N is rapidly transformed by microbes, absorbed by plants, or flushed from the local system during precipitation events (Frost and Hunter 2007). We presumed the loss on collection was 5% (Tadaki *et al.* 1987), while 50% of the amount of grazing was feces (Kikuzawa *et al.* 1979). Subsequently, the amount of grazing of leaf was calculated as 220.0 kg ha<sup>-1</sup> year<sup>-1</sup>, which equaled 6.7% of the leaf litter, 3.28 ton ha<sup>-1</sup> year<sup>-1</sup>.

The C/N ratio of leaf litter decreased from 20 to 12, and interestingly the carbon and N content in leaf litter decreased with maintaining this C/N ratio 12 (figure 6B), which indicated mineralization stage (Takeda 1998). These results suggest that the decomposition of leaf litter of alder immediately moved into the mineralization stage. The decomposition rate of leaf litter of *Alnus* species has been reported to be faster than other non-N<sub>2</sub>-fixer species (Van Cleve *et al.* 1971; Taylor *et al.* 1989; Sharma *et al.* 2008).

The biomass of herbaceous species on the floor of this stand was the largest in the period of the fastest decomposition rate of leaf litter (figure 6A). These results suggest that the vegetation on the floor may play a role in preventing N leaching from this alder stand temporarily and to promoting N accumulation into the stand. It is interesting that the maximum amount of N in herbaceous plants on the floor was almost the same as N<sub>2</sub> fixation per year in this stand (figure 7)

The accumulation of carbon and N actually occurred in the mineral soil of this alder stand after disturbance, but these accumulations were limited to the surface soil (0–0.05 m depth). In temperate deciduous forests, the carbon and N accumulation in mineral soil layer (0–0.7 m depth) was reported as 75.5–90.3 ton C ha<sup>-1</sup> and 4.30–7.46 ton N ha<sup>-1</sup> (Tsutsumi 1989). Nitrogen fixed by *Alnus* species will be transferred to surrounding soils through roots and nodule sections as well as N-rich leaf litter, which will result in increased N availability in forest soils (Sharma *et al.* 2010). Gill and Jackson (2000) reviewed the annual fine root turnover rate as 0.6 to 0.7 year<sup>-1</sup> in temperate-deciduous forests. Aosaar *et al.* (2013) showed that the fine root turnover of *Alnus incana* plantations was 0.54

year<sup>-1</sup>, and the N flux into soil via fine root litter was 5.4 kg ha<sup>-1</sup>, which corresponded to 4.8% of leaf litter N, 112 kg ha<sup>-1</sup>. In the studied alder stand, the whole root biomass was estimated to be 13.3 ton ha<sup>-1</sup>, while the aboveground biomass was estimated to be 51.2 ton ha<sup>-1</sup> by using allometry equation (Komiya *et al.* 2011). When we assumed that the fine root biomass of this alder stand was 2% of the whole tree biomass (Brunner and Godbold 2007), the fine root biomass was calculated as 1.29 ton ha<sup>-1</sup>. In addition, when we used the same fine root turnover rate (0.54 year<sup>-1</sup>) and N concentration (1.246%) as *A. incana* stand reported by Aosaar *et al.* (2013), the N flux into soil via the fine root litter in the studied alder stand was estimated to be 8.7 kg ha<sup>-1</sup>, which corresponded to 10.2% of leaf litter N, 84.9 kg ha<sup>-1</sup>. The estimated fine root biomass in *Alnus* stand (Aosaar *et al.* 2013) were lower than the reported values of the mean fine root biomass in boreal and temperate forests as 5.26 ± 3.21 and 7.75 ± 4.74 ton ha<sup>-1</sup>, respectively (Finer *et al.* 2011). Aosaar *et al.* (2013) discussed that the contribution of fine roots to the soil N increase has been modest due to the constantly low fine root biomass, though there might be underestimation by the in-growth core method.

## 5. Conclusions

There were close relationships between nitrogenase activity and the growth pattern of alder (*Alnus hirsuta* var. *sibirica*), although no differences in nitrogenase activity among tree size (dbh) were obvious. The N<sub>2</sub> fixation rate was estimated at 56.4 kg ha<sup>-1</sup> year<sup>-1</sup>, when the nitrogenase activity was represented by soil temperature, leaf area of alder, and nodule size as presented by the root diameter at nodule base in the conversion rate 3:1. This value corresponded to 66.4% of the N content in leaf litter in a year. It was suggested that, in this stand, alder would have depended on N<sub>2</sub> fixation for a significant part of N absorption. Alder would supply the leaf litter, which have high N content and their rapid decomposition rate, as a result of the lower resorption rate of N compared to other deciduous tree species. These properties of alder will contribute to rapid N accumulation through N<sub>2</sub> fixation into soil. It was suggested that N<sub>2</sub> fixation of alder helped fertilize only the surface soil.

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