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## Growth and nitrogen use characteristics of black locust, an invasive alien species, grown under different light and CO<sub>2</sub> conditions

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### Abstract

Black locust (*Robinia pseudoacacia*) had introduced from north America for vegetation stability after landslides and forest fires, honey plant, etc. however, this species changes vegetation (plagiosere) with its high growth capacity and reproductive capacity. However, adult trees are easy to fall down after infection of root rot, and to regulate this alien species, native root rot fungi were used in Lithuania. Photosynthetic nitrogen (N) use characteristics of this species had been studying *in situ* at open and shade sites under larch plantations for regulation of the growth of this species. No seasonal change in N allocation pattern in leaves was found between open and shade site; N should allocate from N<sub>L</sub> (N in Light Harvesting Chloroplast) to N<sub>R</sub> (N in Rubisco) in a leaf after shedding of top canopy. To simulate increasing atmospheric CO<sub>2</sub> [CO<sub>2</sub>], we studied on growth and evaluation of symbiotic microbe with black locust grown under a free air CO<sub>2</sub> enrichment (FACE). Activities of acetylene reduction of nodule were accelerated in FACE (500 ppm). From these results, black locust will increase its growth rate under nutrient poor sites in increasing [CO<sub>2</sub>] world, therefore, it is essential to develop the management methods which coexist with black locust.

*Key words:* black locust (*Robinia pseudoacacia*), alien species, nitrogen allocation, Acetylene reduction activity, *Rhizobium* sp.

### Introduction

Black locust (*Robinia pseudoacacia*) had been imported from North America for a honey plant as well as stabilizing ground surface after landslide, forest fires, etc. (Sakio 2009). This species often escapes from planted sites and dominates at river-bed, however root is shallow developed and is easy infected root rot fungi, and then will fall down and “attack” around downstream of the river. Moreover, this species usually changes original vegetation (= plagiosere) by its high growth capacity. To keep biodiversity in a forest, we have been discussing how to regulate growth of this species. We hardly regulate this alien species because its vigorous growth capacity (Masaka et al. 2014), and reproductive capacity by two methods of germination (Karaki et al. 2012; Watanabe et al. 2014) and big capacity of sprout (Matsunami et al. 2008; Masaka et al. 2014; Fujita et al. in preparation). These high growth capacities are supported by the highest photosynthetic rate among deciduous broadleaved trees (Koike et al. 2009).

Plant growth is a result of integration between photosynthetic capacity multiply temporal and special arrangement of leaves with environmental plasticity (Larcher 2003). Kitaoka et al. (submitted) reported pinnate compound leaves drastically decrease number of leaflet and its thickness under shade condition in a larch plantation. In Lithuania, they successfully developed an

ideal way of regulation of black locust with use of root rot fungi native to Lithuania (Watanabe et al. 2012).

Our oldest plantation of black locust is located at Japanese larch plantations in the Nakagawa Experiment Forest, Hokkaido University since 1931 and almost no individuals dominate there without any disturbances. Leaves of black locust flush at the complete expansion of short-shoot needles of larch at around mid-June (Kitaoka et al. 2020, submitted). Incident light to forest floor in larch plantations showed drastic seasonal change: relative light (I/I<sub>0</sub>) to forest floor was <45%, around 15%, and <30% during before leaf flush, after leaf flush, and after leaf senescence and shedding, respectively (Kitaoka 2007). Nitrogen (N) in a leaf of regenerated seedlings (oak, carpinus) under larch plantations was allocated to N<sub>C</sub> (Light harvesting chlorophyll protein) after canopy closure of top layer and to N<sub>R</sub> (to Rubisco: CO<sub>2</sub> fixation enzyme in C3 plants) before and after canopy closure (Kitaoka and Koike 2004). Similar N allocation pattern is found in the same crown; leaves at crown surface allocate to N<sub>R</sub> while leaves at inner parts allocate to N<sub>C</sub> (Choi et al. 2021). However, we still have not enough information on seasonal change of the N allocation in leaves of black locust.

On the other hands, atmospheric CO<sub>2</sub> [CO<sub>2</sub>] is continuously increasing at the rate of about 2.2 ppm yr<sup>-1</sup>

during these decade, and reaches to 420 ppm (Mauna Loa 2021). Black locust can grow under infertile soil, which may be attributed to symbiotic microbe in the root system. Photosynthetic rate at elevated CO<sub>2</sub> of red-pine seedlings increased because infected ectomycorrhiza (ECM) *Pisolithus tinctorius* acted as sink for photosynthates (Choi et al. 2005) and a kind of ECM for Japanese larch (Shinano et al. 2007). How about the role of nodular symbionts with black locust under elevated CO<sub>2</sub>, especially nutrient poor condition?

In central Hokkaido, leaf flush of black locust starts around early part of June when upper layer of larch trees has flushed short-shoot needles and is ready for elongation of long-shoot (Kitaoka et al. 2020). N in a leaf is considered to allocate more to N<sub>C</sub> and to N<sub>R</sub> after senescence of needles of the upper canopy like 4 kinds of deciduous broadleaved tree seedling grown in a mature larch forest (Kitaoka and Koike 2004). To access these predictions, we measured seasonal changes in photosynthetic rate for evaluating N allocation of saplings grown at open and shade site (around I/I<sub>o</sub>, 20%) under around a 50-year-old larch plantation. Based on light and N utilization of black locust saplings, we try to show plausible regulation method for regulation this alien species.



**Figure 1.** Photos of black locust in a larch plantation and its leaf morphology, left: grown in a gap in larch plantation, right: pinnate compound leaf of current year shoot of black locust in open site.

## Materials and Methods

### 1. Plant material

#### 1.1. Open and shade sites

Seedlings of black locust (*Robinia pseudoacacia* L.) which invaded into and/ or regenerated at the study sites of 50-year-old larch plantation from avenue trees of the university campus. This species is equipped with pinnate compound leaf and preformed first and second leaves (Figure 1B). Two individuals were selected from each study site (open vs. shade=larch forest floor), and in total 12 individuals were investigated. Seedling size was as follows: height was  $4.1 \pm 0.8$  m, and the diameter at 3 cm from the bottom was between  $7.3 \pm 2.1$  cm.

Test shoots were chosen at around 1.5 m and the top leaflet of pinnate compound leaf of the sample trees without any disease and insect grazing. After determination of gas exchange rates, age of samples was determined by analysis of annual rings (seedling age of shade and open samples was 3 and 2 years old, respectively).

### 1.2. FACE

Two-year-old saplings were grown in a free air CO<sub>2</sub> enrichment (FACE) site of at the Sapporo Experiment Forests the Field Science Center for Northern Biosphere, Hokkaido University. Soil was typical brown forest soil. There were 6 rings (H=5.5m D=6.5m); 3 of them were fumigated with elevated CO<sub>2</sub> at 500ppm while the rests were ambient CO<sub>2</sub> (370~380 ppm as of 2006).

## 2. Measurements

### 2.1. Photosynthesis traits and N allocation

Seasonal changes in the net photosynthetic rates at light saturation and ambient [CO<sub>2</sub>] (380 ppm) (P<sub>sat</sub>) were measured for the same position of leaflet of a pinnate compound leaf of each individual sapling grown under open and shade sites. The P<sub>sat</sub> was measured from June to the beginning of November, i.e., the measurement was performed five times at mid-June, latter part of July, late August to early September, late September to early October and late October to early November. After gas exchange measurement, LMA (leaf area per its dry mass; g/m<sup>2</sup>) and chlorophyll contents (Shinano et al. 1996) were determined.

The photosynthetic rate was determined with a portable photosynthesis transpiration system (LI-6400, LiCor, Nebraska, U.S.A.) with a LED source attached with blue light (LI-6400-02). P<sub>sat</sub> was determined under ambient CO<sub>2</sub>, steady leaf temperature (25°C), and photosynthetic photon flux density (PPFD) around 1,400 μmol m<sup>-2</sup> s<sup>-1</sup> as previously determined (Choi et al. 2017). The maximum photosynthetic rate at light and [CO<sub>2</sub>] saturation (P<sub>max</sub>) was determined at [CO<sub>2</sub>] 1700 ppm, PPFD around 1500 μmol m<sup>-2</sup> s<sup>-1</sup> and leaf temperature of 25°C.

The value of N<sub>C</sub> was calculated from the amount of chlorophyll based on the method of Evens and Seemann (1987). The calculation method of the percentage of N allocation is as follows:

$$\cdot N_C (\%) = (\text{Chl A} + \text{Chl B}) \times 0.519 / N_{\text{area}} \times 100$$

$$\cdot N_E (\%) = J_{\text{max}} / 1257.360 N_{\text{area}} \times 100$$

$$\cdot N_R (\%) = V_{\text{cmax}} / 129.375 N_{\text{area}} \times 100,$$

where, N<sub>C</sub>: Nitrogen content of LHC, PS I and PS II; N<sub>E</sub>: Electron transport proteins except N<sub>C</sub> plus the nitrogen content of carbon cycle proteins except rubisco; N<sub>R</sub>: Nitrogen content of rubisco; Chl A (mmol m<sup>-2</sup>): Molar concentration of chlorophyll a; Chl B (mmol m<sup>-2</sup>): Molar concentration of chlorophyll b; N<sub>area</sub>: Nitrogen content per area.

### 2.2. Estimation of N<sub>2</sub> fixing

The N fixation rate in *R. pseudoacacia* was determined by acetylene reduction assay (ARA) (Peterson and Burris 1976; Choi et al. 2017). Approximately 2.5 g of nodules with fine roots were

obtained from the same trees used for measuring photosynthetic rate, and was placed immediately in the syringe. Then, 10% (v/v) of the air in the syringe was removed and replaced with an equal volume of acetylene ( $C_2H_2$ ) (99.9%). After 30 min, 10 ml of gas was removed from the syringe and injected into a gas chromatograph to measure the production of ethylene from the acetylene (G-3000, Hitachi, Tokyo, Japan).

## Results and Discussion

### Leaf morphology, gas exchange and N allocation in leaf

Light condition of forest floor from June to early September was around  $45 \mu\text{mol m}^{-2}\text{s}^{-1}$  and relative value ( $I/I_0$ ) was around 25% during July to late August, and the  $I/I_0$  increased 45~80% from mid-September to early November. The  $I/I_0$  trend reflected leaf phenology of canopy of larch trees; namely short-shoot needles develop by mid-June and succeeding long-shoot needles develop, and start to shed needles at around early October (Kitaoka et al. 2020).

At shade sites, shoot development of black locust was clearly suppressed, and the leaves had a lower LMA with undeveloped palisade layer. These responses are similar to typical shade-intolerant deciduous broad-leaved tree species in cool temperate forests in Japan

$P_{\text{max}}$  at open sites reached maximum value at July and decreased from August toward autumn. At shade sites, little changes were observed from June to September but showed a decreasing trend from September to leaf-fall in October. At forests of cool temperate regions, N is one of the factors limiting plant growth. Black locust has a relatively high value of  $N_{\text{area}}$  as compared with other deciduous broad leaved tree species (oak, magnolia, carpinus, etc.) grown in similar mature larch plantations (Kitaoka and Koike 2004). This result implies that black locust grows under rich nitrogen conditions as it has a symbiotic relationship with N-fixative micro-organisms. This is agreeable from the results of low N-remobilization rates and PNUE at shade sites (Kitaoka et al. submitted).

Some tree species in larch plantations showed unique N allocation pattern to photosynthetic organs. N allocation percentages in a leaf of shade-tolerant species (Ssiori-cherry and carpinus) grown under  $I/I_0 = 18\%$ – $20\%$  of unmanaged larch stands; the two shade-tolerant species can allocate N to  $N_C$ ,  $N_E$ ,  $N_R$ , etc., in accordance with incident light to the forest floor. For example, Ssiori-cherry seedlings allocate about 8% N to  $N_C$  before canopy closure and about 26% during canopy closure, then about 12% after canopy opening.

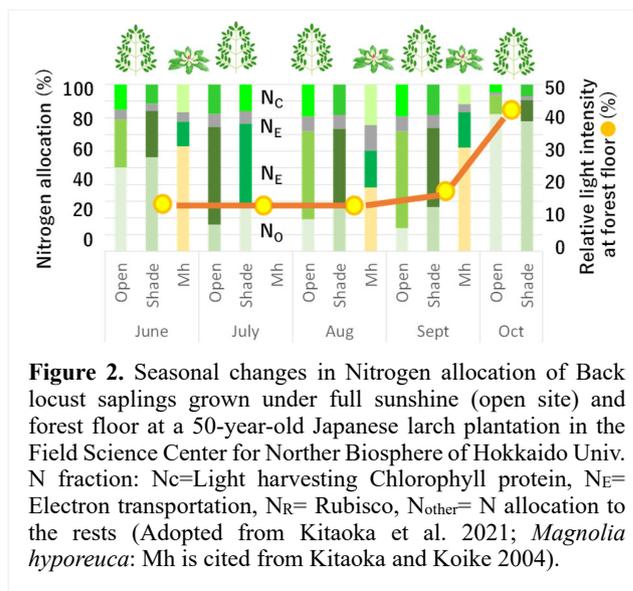
In contrast, no change was found in N allocation pattern in a leaflet of black locust by mid-September. Irrespective of our prediction, black locust seedlings allocated about 18% of leaf N to  $N_C$  independent of light conditions. This indicates that the pattern of N allocation of black locust is different from general knowledge obtained from other tree species (Kitaoka and Koike 2004, Kitaoka 2007), namely, black locust grown in weak light condition could not allocate N to  $N_C$ .

No difference was found in the N allocation pattern

of black locust between the two sites during the growth period, although light conditions were drastically changed in shade sites from mid-August until leaf yellow in November.

### Growth at elevated $CO_2$

The acetylene reduction activity (ARA) of symbiosis nodule with *R. pseudoacacia* grown at elevated [ $CO_2$ ] was significantly greater than for that grown at ambient [ $CO_2$ ] (after Choi et al. 2017). The ARA at ambient



**Figure 2.** Seasonal changes in Nitrogen allocation of Black locust saplings grown under full sunshine (open site) and forest floor at a 50-year-old Japanese larch plantation in the Field Science Center for Northern Biosphere of Hokkaido Univ. N fraction:  $N_C$ =Light harvesting Chlorophyll protein,  $N_E$ =Electron transportation,  $N_R$ =Rubisco,  $N_{\text{Other}}$ =N allocation to the rests (Adopted from Kitaoka et al. 2021; *Magnolia hyporeuca*: Mh is cited from Kitaoka and Koike 2004).

[ $CO_2$ ] was  $12.2 \mu\text{mol } C_2H_4 \text{ g}^{-1} \text{ nodule h}^{-1}$ , and the ARA nodule grown at elevated [ $CO_2$ ] was  $15.0 \mu\text{mol } C_2H_4 \text{ g}^{-1} \text{ nodule h}^{-1}$ , which was not significant ( $P < 0.05$ ). Black locust can live with several microbes in soil even under infertile soil condition (Li et al. 2021). Elevated  $CO_2$  world will continue and essential role of symbiotic microbes (e.g., *Rhizobium* sp.) in root of black locust will become more important for offering N resources to vegetation via black locust and may change vegetation more greatly in nutrient poor sites. Shinano et al. (2007) revealed an increase of sink activities with ECM in Japanese larch seedlings grown under elevated [ $CO_2$ ].

However, we observed a photosynthetic down-regulation of black locust in response to elevated [ $CO_2$ ]. This may be associated with reduction of N and Rubisco content, and also changed N allocation patterns in the leaves under elevated  $CO_2$  with the FACE ([ $CO_2$ ] 500 ppm) (Table 1). Therefore, we should develop more efficient methods for regulation of black locust like Lithuania (Watanabe et al. 2012).

### Conclusion

Light-saturated photosynthesis ( $P_{\text{sat}}$ ) of black locust seedlings were suppressed under weak light environments, such as  $I/I_0 \leq 25\%$ . Acclimation capacity of black locust to low light environment may be small compared to other tree species, because of almost no change in the N allocation in photosynthesis system (especially to  $N_C$ ,  $N_R$ ) was observed. As compared with other reported species, low N-remobilization of Black locust may be due to symbiotic N-fixing microbes.

**Table 1.** Photosynthetic parameters of Black locust saplings at control and FACE

[CO <sub>2</sub> ]	Rubisco/N	Chl/N	Vcmax/N	Jmax/N	Jmax/Vcmax
Amb	1.37(0.23)	0.09(0.01)	32.25(2.48)	29.27(4.14)	0.97(0.03)
FACE	1.02(0.19)	0.11(0.01)	35.42(3.22)	33.82(5.31)	0.93(0.07)
P value	0.02	0.04	ns	ns	ns

Abb: Amb: ambient [CO<sub>2</sub>] = about 380 ppm, FACE: [CO<sub>2</sub>] = about 500 ppm. In parenthesis, figures mean SD. \*Unit: Rubisco/N [g m<sup>-2</sup>/g m<sup>-2</sup>], Chl/N [g m<sup>-2</sup>/g m<sup>-2</sup>], Vcmax/N [μmol m<sup>-2</sup>s<sup>-1</sup>/g m<sup>-2</sup>], Jmax/N [μmol m<sup>-2</sup>s<sup>-1</sup>/g m<sup>-2</sup>], Jmax/Vcmax [μmol m<sup>-2</sup>s<sup>-1</sup>/μmol m<sup>-2</sup>s<sup>-1</sup>] Adopted from Choi et al. (2017)

Therefore, black locust should live in open sites for maximizing photosynthesis. According from these results, we suggest that black locust in a forest should be managed as follows: we should carefully observe the stand structure and growth condition of black locusts that have invaded the area, and then, individuals grown near or in canopy gaps should be eliminated to efficiently suppress further invasion.

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