

Temporal changes in nitrogen acquisition of Japanese black pine (*Pinus thunbergii*) associated with black locust (*Robinia pseudoacacia*)

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Abstract: The alien woody legume, black locust (*Robinia pseudoacacia*), has invaded Japanese black pine (*Pinus thunbergii*) forests located in Japan's coastal plain and hill regions where gaps are formed in pine forests after nematode infestation. Nitrogen fixation by legumes accelerates N cycling in forest ecosystems. We studied temporal change in the annual tree-ring resolution N stable isotope composition ($\delta^{15}\text{N}$, a per mil deviation of $\delta^{15}\text{N}/^{14}\text{N}$ ratio, relative to atmospheric N_2 , $\delta^{15}\text{N}=0\text{‰}$) at two natural locations of Japanese black pine forest with black locust that differed in the time since black locust establishment (Shohnai in northeast and Kita-Kyushu in southwest Japan). Analyzed tree-rings covered the period from 1990/1992 to 2009. N acquisition by Japanese black pine from black locust N input to the soil was evidenced by temporal shifting of N stable isotope composition on the annual pine tree rings. With progressive development of the forest stand, $\delta^{15}\text{N}$ values of earlier tree-rings ($\delta^{15}\text{N}$ of -5‰) from black pine associated with black locust shifted towards values similar to those of black locust $\delta^{15}\text{N}$ values nearly to -1‰), which suggests acquisition of N by N_2 fixation (Shohnai site). In contrast, in a forest where black locust had settled for two or three generations, in a black pine stand (Kita-Kyushu site), longer periods of N enrichment in the soil were reflected in the elevated tree-ring $\delta^{15}\text{N}$ values of newly established black pine trees. Based on tree-ring $\delta^{15}\text{N}$ data from the

Shohnai site, we determined that about 10 years after black locust establishment, soil N had already been enriched by black locust N, this, in turn, contributed to N fertilization of surrounding trees in mixed stands.

Keywords: $\delta^{15}\text{N}$ natural abundance, Japanese black pine, black locust, nitrogen acquisition, tree-ring

Introduction

The nitrogen-fixing tree black locust (*Robinia pseudoacacia*) is the second most abundant deciduous tree species in the world (Malcolm et al. 2008). This competitive, early-successional species native to the Appalachian uplands in USA has rapidly invaded several countries (Malcolm et al. 2008). Nitrogen-fixing species can impact the structure and function of plant communities by contributing a high annual budget of N-rich litter of up to $100 \text{ kg}\cdot\text{ha}^{-1}\cdot\text{a}^{-1}$ (Binckley et al. 1985; Moon and Haruki 1999; Baddeley et al. 2000; Schulze 2000; Rice et al. 2004). Invasion of these species elevates soil N concentration and increases rates of subsequent N cycling, including mineralization and nitrification (and probably denitrification) in forest ecosystems (Binckley et al. 1985; Kawata 1987; Schulze et al. 1991; Rice et al. 2004; Malcolm et al. 2008).

In Japan, black locust was first introduced in the middle of the 19th century to stabilize Japanese black pine (*Pinus thunbergii*) plantations on coastal sand dunes along the Sea of Japan where N-poor habitat prevails. The legume species has recently invaded gaps caused by the spread of pine nematode (Taniguchi et al. 2007). Taniguchi et al. (2007) reported that the invasion reduces N acquisition by the associated black pine due to elevated concentration of nitrate-N. Temporal changes in the process of N acquisition by black locust and associated black pine are expected to be recorded in the annual rings and to represent past interactions (Poulson et al. 1995; Sauer et al. 2004; Savard 2010).

Use of the naturally abundant N isotope composition ($\delta^{15}\text{N}/^{14}\text{N}$ ratio), commonly designated as $\delta^{15}\text{N}$, (a per mil variation relative to atmospheric nitrogen, where $\delta^{15}\text{N}=0$) has been

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recognized as a method of tracing N nutrition in studies of forest ecosystems (Natehoffer and Fry 1988; Gebauer and Shulze 1991; Robinson 2001; Hart and Classen 2003; Lopez et al. 2010; Mizota et al. 2011). Under humid and temperate climatic conditions, $\delta^{15}\text{N}$ values for wood of black locust are expected to be around zero per mil because limited N isotope fractionation results from biological nitrogen fixation (Robinson 2001). Black pine grown in humid and temperate climates such as those of central to southern Japan has $\delta^{15}\text{N}$ values in a narrow range for any geographic region. This reflects the sole N source (NH_4^+ and NO_3^- -N) in meteoric precipitation in open soil systems (Lopez et al. 2010; Mizota et al. 2011). In forest gaps newly formed after nematode disease outbreaks, e.g. those in forests along the Sea of Japan coast, black locust invades, resulting in the formation of mixed forests (Taniguchi et al. 2007). In any forest ecosystem, when other nutrients are not limiting, increases in N availability can enhance tree growth (Bouillet et al. 2008). Black locust is a fast growing species with high biomass production. Because of its N fixation ability it can be used to reduce the demand for external fertilization, especially if the timing for maximum N transfer from black locust to black pine is determined. The aim of this study was to investigate N acquisition by Japanese black pine associated with black locust by using $\delta^{15}\text{N}$ analyses of annual tree rings (resolution of one year) at two locations in Japan. We discuss temporal changes in $\delta^{15}\text{N}$ in relation to the growth of associated N-fixing legumes.

Materials and methods

Description of the study sites

Two study sites were selected to represent different durations of black locust establishment in mixed forest with black pine. Recently invaded forest was represented by the Shohnai site in northeast Japan, while longer black locust presence was represented by the Kita-Kyushu site in southwest Japan. Soils at both study sites were classified as Dystric Regosols (FAO/UNESCO system) developed on coastal dune sands. Mean annual air temperature and annual rainfall at the nearest meteorological stations (Sakata and Fukuoka, respectively) are 12.1 and 16.2°C, and 1857 and 1604 mm, respectively. A brief description of the sites is given below.

Shohnai site

This study site is representative of temperate-humid regions in Yamagata Prefecture, northeast Japan. The Shohnai (38°49'14"N, 139°47'47"E) coastal areas in northern Yamagata are subject to strong seasonal winds during winter. For more than 150 years, black pine has been planted along the coast to prevent sand movement, which causes serious damage to agricultural crops in back-dune areas. The site selected for sample collection was located on the west-facing slope of a coastal sand dune where black locust recently (early 1990s) invaded clearings in black pine forests. The growth of black locust is faster than that of the associated black pine (Kawata 1987; Taniguchi et al.

2007) and contributes to the increase of N input to soils, providing a new N source for the surrounding black pine. The densities of *Pinus thunbergii* and *Robinia pseudoacacia* on the study area was estimated at 2,100 and 2,400 trees per hectare, respectively.

Kita-Kyushu site

This study site represents the warm and humid regions in Fukuoka in southwest Japan. The site (33°39'53" N, 130°21'21" E) was along the Genkai Sea at the location of the flat or nearly flat National Reserve of Marine Park. This area was also invaded by black locust after clearings formed in black pine forests infested by pine nematodes, but invasion occurred about 20 years earlier here (early 1970s) than at the Shohnai site (Gyokusen et al. 1991). At this site, decaying large trunks of black locust are common, suggesting that black locust has occurred here for at least two generations. At this site there was no pure black locust stand. The densities of *P. thunbergii* and *R. pseudoacacia* during the present study site were estimated at 1760 and 4750 trees per hectare, respectively (Gyokusen et al. 1991).

Selection of annual tree ring samples

Representative trees were carefully selected based on observations of their spatial distribution. Tree cores were collected at both sites from Japanese black pine (soft wood) and black locust (porous wood) trees, using an increment borer (diameter of 12 mm, Haglof, Langsele, Sweden). Core samples were collected as follows: One core sample from a pure black pine stand, three core samples from three black pine trees in a mixed forest with black locust (at the Kita-Kyushu site only one sample was collected) and one core sample from a pure black locust stand. The increment borer was inserted into the tree from one side to the other side of the trunk (thus two sides were collected at once with the pith included). All samples were collected at breast height (1.3 m above ground level). The cores were taken to a laboratory for sampling of annual tree-rings (resolution of one year) to quantify $\delta^{15}\text{N}$ values. One of the core samples from black pine in the mixed forest was analyzed from 1992 while the other two were analyzed from 1999 to 2009. The tree ring series ranged from the first year (1990/1992) to 2009 except for the sample from a pure black pine stand from Shohnai that was approximately 42 years old. All analyzed tree rings corresponded to the sapwood area. Each tree ring was systematically dissected using a sharp, high-quality, stainless blade, and dried at 55 °C overnight. Each ring sample was then cut into small pieces using a nail cutter.

Nitrogen isotope analysis

Extraction of labile N was not carried out because there were negligible changes in $\delta^{15}\text{N}$ values in tree rings after extraction of labile N in previous studies (Couto-Vazquez and Gonzales Prieto 2010; Doucet et al. 2011; Lopez et al. 2011). $\delta^{15}\text{N}$ values for dry ring samples were estimated using a CF-IRMS (continuous-flow type mass spectrometer: Iso Prime mass spectrometer, GV Instruments, UK) installed at the Faculty of Science, Okayama

University. The evolved gas was first passed through a column packed with solid CaO-NaOH reagent (soda lime) to eliminate excess CO₂ and then subjected to gas chromatography to separate N₂ followed by mass spectrometric measurement of ¹⁵N/¹⁴N ratios. The isotopic compositions of samples were expressed relative to atmospheric N₂ ($\delta^{15}\text{N}=0$) on scales normalized to the known $\delta^{15}\text{N}$ values of laboratory working standards for glycine ($\delta^{15}\text{N}=-0.3\text{‰}$), which was normalized to L-glutamic acid distributed as USGS-40 ($\delta^{15}\text{N}=-0.2\text{‰}$) by SI Science Inc., Japan. The working standard was analyzed after every eight to ten samples during CF-IRMS runs to assess the replicability of the isotope measurements and normalization. One pulse of pure N₂ reference gas from a tank reservoir ($\delta^{15}\text{N}=-2.5\text{‰}$) was discharged into the IRMS at the beginning of each chromatogram for both standards and samples. The accuracy obtained for standards and samples during the overall analytical procedure was better than $\pm 0.2\text{‰}$ for sample sizes of $\geq 10 \mu\text{g N}$.

Results and discussion

Temporal changes in tree ring nitrogen isotope composition

It is well documented that tree-rings of Japanese black pine retain their intrinsic record of N acquisition (Lopez et al. 2010; Mizota et al. 2011; Lopez et al. 2011). Temporal changes in nitrogen isotope composition of tree-rings grown after 1992/1994 from Shohnai and Kita-Kyushu sites are shown in Fig. 1 and 2, respectively. Clear differences were recorded for $\delta^{15}\text{N}$ values of black locust and black pine.

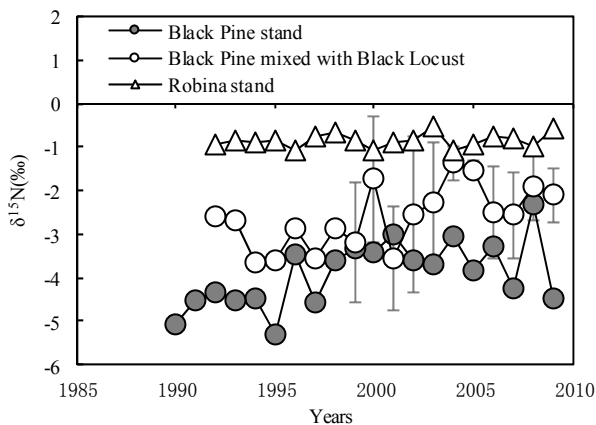


Fig. 1: Temporal changes in $\delta^{15}\text{N}$ values of annual tree-rings from Shohnai site. The standard deviation of black pine in the mixed stand corresponds to three core samples per tree-ring. Higher variation in $\delta^{15}\text{N}$ value of ring samples from black pine, relative to those of black locust is also noticeable. For detailed explanation of the plots described in the legends, see the section Materials and methods.

Shohnai site

$\delta^{15}\text{N}$ values of black locust tree rings from the pure stand varied in a narrow range from -1.1‰ to -0.5‰ (average = -0.9‰ , $n=18$). Such values approximate those of atmospheric N₂ ($\delta^{15}\text{N}=0$). Because limited N isotope fractionation is associated with N

fixation (Robinson 2001), this result confirms the substantial contribution by N fixation in this woody legume. $\delta^{15}\text{N}$ values of tree rings from the pure black pine stand ranged from -5.3‰ to -2.3‰ , averaging -3.9‰ . Nitrogen isotope values were similar to those of inorganic nitrogen input from meteoric precipitation as observed at a nearby site ($\delta^{15}\text{N}=-5.5\text{‰}$ to -1.7‰) (Fukuzaki and Hayasaka 2009) during May 2001 to November 2002.

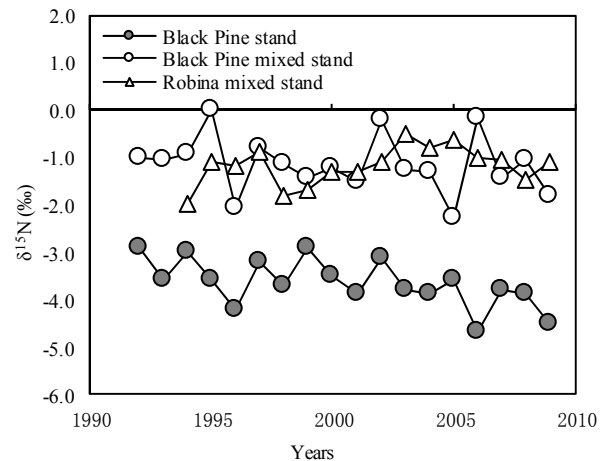


Fig. 2: Temporal changes in $\delta^{15}\text{N}$ values of annual tree-rings from Kita-Kyushu site. The $\delta^{15}\text{N}$ values of ring samples from black pine are more variable, relative to those of black locust. For detailed explanation of the plots described in the legends, see the section Materials and methods.

$\delta^{15}\text{N}$ values for tree rings from black pine in the mixed stand were markedly higher than those from the pure stand. A steady increase was observed from 1992 (-2.6‰) to 2009 ($-2.1\text{‰} \pm 0.6\text{‰}$). Tree ring $\delta^{15}\text{N}$ values in 1999 and 2004 were ($-3.2 \pm 1.4\text{‰}$) and ($-1.4 \pm 0.4\text{‰}$), respectively. The $\delta^{15}\text{N}$ values for 2002 to 2009 approached those of the associated black locust.

$\delta^{15}\text{N}$ values of tree rings of black pine for the first and second years (1992 to 1993) in the mixed stand showed clearly higher values (-2.7‰ and -2.6‰) than for the corresponding years for the pure black pine stand (-4.5‰ and -4.3‰). This could indicate that somewhat elevated $\delta^{15}\text{N}$ values resulted from the N input from past invasion of black locust prior to the present stand of Japanese black pine (Fig. 1).

Kita-Kyushu site

The overall temporal trend of $\delta^{15}\text{N}$ values at the Kita-Kyushu site was different from that observed for the Shohnai site. From 1994, $\delta^{15}\text{N}$ values of black locust tree rings in the mixed stand ranged from -2.0‰ to -0.5‰ , averaging -1.2‰ .

$\delta^{15}\text{N}$ values for black pine tree rings from a pure stand ranged from -4.7‰ to -2.9‰ for the period 1992–2009, averaging -3.7‰ . These values were nearly identical to those for the Shohnai site ($\delta^{15}\text{N}=-3.9\text{‰}$). In contrast, $\delta^{15}\text{N}$ values for tree rings from black pine in the mixed stand showed higher values that ranged from -2.3‰ to 0 and averaged -1.2‰ for the entire growth period, suggesting incorporation of N derived from N₂ fixation from the very first year of Japanese black pine establishment in this area. This indicates that several generations

(more than 30 years) of black locust in this area have enriched soil N to the point that it is readily available to newly established Japanese black pine (Fig. 2).

Potential use of nitrogen stable isotope composition in analysis of the acquisition of nitrogen in mixed forests

From the temporal fluctuations and elevated isotope values observed in tree rings of black pines on both sites, it is clear that the main cause of this change is the acquisition of N derived from N₂ fixation through association with black locust. To a lesser degree, however, the roles of processes such as mineralization, nitrification and denitrification must also be considered.

It is well known that woody legumes fix variable amounts of atmospheric N₂. Estimates range from nearly 0 to 204 kg·ha⁻¹·a⁻¹ (Bouillet et al. 2008). The highest value reported for black locust was 110 kg·ha⁻¹·a⁻¹ (calculated contribution of atmospheric N₂ relative to whole N absorbed from the environment = 90%) (Danso et al. 1955). The nitrogen isotope analysis for annual tree rings can be applied to forest management practices in which woody legumes are mixed with other species and, as in the case of this study, can enhance growth of black pine forests that provide windbreaks in coastal areas along the Sea of Japan. However, the competition between the two species could counterbalance the positive effect of black locust on the growth of black pine as reported for red alder–Douglas fir forest (Binckley et al. 1985). Malcolm et al. (2008) reported that black locust was completely removed prior to the reestablishment of pine-oak forest because of the enhanced growth of these species to soils enriched by high levels of N resulting from the prior presence of black locust. However, this is apparently not the case for black pine since it was not affected by high levels of N in the soil in forest receiving high input of avian N (Lopez et al. 2011; Mizota et al. 2011).

Taniguchi et al. (2007) reported that black pine relies on ectomycorrhizal (ECM) colonization for N uptake from the soil. Since the ECM community changes because of N enrichment in the soil, the N enrichment effect of black locust on black pine trees is negative. Nevertheless, black pine trees exposed to high inputs of avian N grow without limitation in other areas of Japan (Lopez et al. 2011; Mizota et al. 2011), suggesting that even when ECM symbiosis is modified, black pine trees can uptake available N directly from the soil. Changes in ECM functions related to increases in N input remain, however, a topic for future studies.

Our study results contribute to understanding the positive role of black locust in forest management practices for black pine in coastal areas of the Sea of Japan. Elevation of δ¹⁵N values in tree rings of Japanese black pine resulting from biological N fixation from associated black locust saturates within ca. 10 years after the black locust invasion (Fig. 1). This estimate enables improved scheduling of the removal of black locust during the early growth stages of black pine plantations. Such practices have not yet been implemented, despite the role of black locust in N fixation under low soil fertility conditions.

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