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REVIEW

Responses of symbiotic N_2 fixation in *Alnus* species to the projected elevated CO_2 environment

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Abstract

Key message **Nitrogen fixation in *Alnus* species in response to elevated CO_2 may depend on the presence of non-N_2-fixing tree species in addition to soil conditions.**

Abstract *Alnus* is a major genus of actinorhizal plants. Symbiosis with *Frankia* allows the *Alnus* species to fix nitrogen (N) at the rate of several to 320 kg N ha^{-1} $year^{-1}$ with a nodule biomass of 16–480 kg ha^{-1}. *Alnus* species ensures an effective supply of N to soils because of the high N content of leaf litter, rapid decomposition rate, and the influx of herbivorous insects. In addition, the association between regenerated endozoochorous species and *Alnus hirsuta* suggests that N_2 fixation in *Alnus* species influences the distribution patterns of regenerated plants as well as improve soil fertility. N_2 fixation by the *Alnus*–*Frankia* symbiotic relationship may be positively associated with elevated carbon dioxide (CO_2) levels. Nodule biomass increased under elevated CO_2 due to enhanced plant growth, rather than changes in biomass allocation. The inhibitory effect of high soil N on nodulation was retained under elevated CO_2, and the effects of elevated CO_2 on N_2 fixation depended on soil P availability, drought, and many other abiotic and biotic factors. Recent free-air CO_2 enrichment experiments have demonstrated increased N_2 fixation in *A. glutinosa* exposed to elevated CO_2 in mixed-species stands containing non-N_2-fixers but not in monocultures, suggesting that N_2 fixation depends on an association with non-N_2-fixing tree species. Because elevated CO_2 can alter the N and P contents and stoichiometry of plants, it will be necessary to evaluate N allocation and accumulation of biomass when investigating the response of *Alnus* species to future global climate change.

Keywords Actinorhizal plants · *Frankia* · Nodule biomass · Soil nutrients · Stoichiometry

Introduction

Biological nitrogen (N) fixation is an important pathway to input new N into terrestrial ecosystems (Vitousek and Walker 1987). Two types of root-nodule symbioses exist between higher plants and N_2-fixing soil bacteria, such as legume–*Rhizobium* and actinorhizal plant–*Frankia* symbioses (Pawlowski and Sprent 2008). Bacteria induce the formation of nodules on plant roots during both interactions. In the case of actinorhizal symbiosis, Gram-positive actinomycetous soil bacteria in the genus *Frankia* induce the formation of root nodules in >200 dicotyledonous plants from eight families (Dawson 2008; Gtari et al. 2013). All of these host plants are perennial dicotyledons, and all except the genus *Datisca* are trees or shrubs. Although leguminous tree species are dominant symbiotic N_2-fixers in tropical regions, actinorhizal woody plants and *Frankia* play an important role in the N cycle in temperate and boreal forest ecosystems (Huss-Danell 1997). Recent research on actinorhizal plant—*Frankia* symbioses have focused on phylogenetic and genomic analyses regarding

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the use of transgenic actinorhizal plants (Kucho et al. 2010; Normand 2013).

Among actinorhizal plants, which are very diverse, those from the genus *Alnus* are of particular interest (Pourhassan et al. 2015). Forty-seven *Alnus* species are found worldwide (Pawlowski and Newton 2008), 16 of which grow naturally in Japan (Uemura and Sato 1975). The *Alnus–Frankia* symbiotic relationship has been used to revegetate deteriorated wildlife habitats and rehabilitate N_2-deficient disturbed areas (Sharma et al. 2002; Hanley et al. 2006) to enhance the growth of commercial coniferous trees (Vogel and Gower 1998; Son et al. 2007) and short-rotation plantings used for biomass energy (Eriksson and Johansson 2006; Claessens et al. 2010; Uri et al. 2011; Hytönen and Saarsalmi 2015). The range of N_2 fixation rates reported for the *Alnus–Frankia* symbiosis coincides with that of the legume–*Rhizobium* symbiosis (Hibbs and Cromack 1990; Lambers et al. 2008; Noh et al. 2010).

In addition, the *Alnus–Frankia* symbiotic relationship is attracting interest due to its responses to elevated atmospheric carbon dioxide (CO_2) concentrations (Hungate et al. 2003). Increasing CO_2 is an important factor influencing global climate change (IPCC 2007, 2013). Elevated CO_2 increases long-term forest net primary productivity (Zak et al. 2011), except under nutrient-limiting conditions (Leuzinger and Hättenschwiler 2013), and forest ecosystems are usually N_2 limited (Vitousek and Howarth 1991; Wang et al. 2010). Although carbon (C) becomes more available to plants in an elevated CO_2 environment, plants also require other resources to sustain primary production, including N, phosphorus (P), and micronutrients obtained from the soil (Pourhassan et al. 2015). Therefore, nutrient availability, particularly that of soil N, may strongly limit responses to elevated CO_2 by woody plants undergoing increased photosynthetic and growth rates (Luo et al. 2004; Hyvönen et al. 2007; Norby et al. 2010; Zak et al. 2011; Sigurdsson et al. 2013). Exogenous N input into forest ecosystems may be required to maintain enhanced growth under these conditions (Johnson 2006). Symbiotic and/or heterotrophic N_2 fixation by legumes and actinorhizal plants may be a potential source of N to sustain increased N uptake by non-N_2 fixers due to high rates of forest productivity under an elevated CO_2 environment (Vitousek et al. 2002; Finzi et al. 2007). Many studies on legumes have been conducted, and excellent reviews have been published (Ainsworth and Long 2004; Ainsworth and Rogers 2007; Rogers et al. 2009).

A number of abiotic and biotic factors affect the free-living and symbiotic properties of the *Frankia*–actinorhizal symbiosis, including moisture, aeration, temperature, pH, organic matter, inorganic chemicals, and the distributions of *Frankia*, and mycorrhizal fungi; excellent reviews have summarized these effects (Huss-Danell 1997; Dawson 2008; Valdés 2008; Põlme et al. 2014). The growth responses of legumes to elevated CO_2 are occasionally constrained by factors other than N availability, such as P availability and water conditions (Hungate et al. 2004; Reverchon et al. 2012). Understanding how *Alnus* species respond to elevated CO_2 is of great ecological and economical importance (Pourhassan et al. 2015). This review primarily focuses on the details of the interactive effects between projected elevated CO_2 in the near future and other factors such as N, P, and drought on growth and N_2 fixation in *Alnus–Frankia* symbiosis. The first section presents a review on determining nodule biomass and N_2 fixation rates under the present conditions.

Current *Alnus–Frankia* N_2 fixation ability

Nodule biomass in *Alnus* stands

Nodule biomass is an essential tool to estimate N_2 fixation at any scale (Aosaar et al. 2013). Nodules are formed on actinorhizal plants when lateral roots are infected by *Frankia*. In some hosts such as *Alnus* species, *Frankia* infects roots via root hairs (intracellular infection) or by intercellular penetration (Wall and Berry 2008; Tromas et al. 2013). Nodules can be perennial clusters of modified lateral roots and may grow to a large size (Huss-Danell 1997). The size and mean weight of *Alnus* species nodules increase with the tree diameter within a naturally established stand (Uliassi and Ruess 2002; Tobita et al. 2010a) and increase with stand age in a plantation (Aosaar et al. 2013). The nodule size affects nitrogenase activity (NA) because nodules have varying amounts of non-N_2-fixing tissue with increasing size (Sharma and Ambasht 1984; Hurd et al. 2001). This fundamental information about nodule size distribution is useful to estimate N_2 fixation in *Alnus* stands. However, NA, at least in young *Alnus* species plants, is related to nodule biomass (Gordon and Wheeler 1978) and not nodule number (Dawson and Gordon 1979).

Several studies have attempted to estimate nodule biomass in *Alnus* stands living in managed plantations and in naturally established populations. Nodule biomass varies depending on stand age, species composition, tree size, stand density, and soil nutrient concentrations (Binkley 1981, 1982; Bormann and Gordon 1984; Sharma and Ambasht 1986; Binkley et al. 1992; Uliassi and Ruess 2002; Lee and Son 2005; Son et al. 2007) and has been estimated to range from 16 to 480 kg ha^{-1} (Table 1; Binkley 1981; Hurd et al. 2001). No clear association has been found between nodule biomass and stand age but nodule biomass tends to increase early until a stand is 10–15 years old (Fig. 1a). Variations in below-ground biomass estimates are higher than those of above-ground

Table 1 Estimates of nodule biomass in *Alnus* stands

Species	Nodule biomass (kg ha^{-1})	Stand age (years)	Tree density (trees ha^{-1})	References
Alnus glutinosa	454	5–20		Akkermans and van Dijk (1976)
Alnus hirsuta	88	17	1114	Tobita et al. (2010a)
Alnus hirsuta	179	27	1960	Son et al. (2007)
Alnus hirsuta	95	27	1130	Son et al. (2007)
Alnus hirsuta	220	38	700	Lee and Son (2005)
Alnus incana ssp. *incana*	170	5	12660	Aosaar et al. (2013)
Alnus incana ssp. *incana*	160	10	7400	Aosaar et al. (2013)
Alnus incana ssp. *incana*	310	17	5100	Aosaar et al. (2013)
Alnus incana ssp. *incana*	44	2		Huss-Danell and Ohlsson (1992)
Alnus incana ssp. *incana*	110	3	40000	Rytter (1989)
Alnus incana ssp. *incana*	230–480	7		Rytter (1989)
Alnus incana ssp. *incana*	150	30		Johnsrud (1978)
Alnus incana spp. *rugosa*	65		19120	Hurd et al. (2001)
Alnus incana spp. *rugosa*	58			Younger and Kapustka (1983)
Alnus nepalensis	300–420	15–20		Sharma et al. (2010)
Alnus nepalensis	457	7	715	Sharma and Ambasht (1986)
Alnus nepalensis	149	56	435	Sharma and Ambasht (1986)
Alnus rubra	250–325	55	3000	Binkley et al. (1992)
Alnus rubra	16	2		Kim (1987)
Alnus rubra	121	58		Kim (1987)
Alnus rubra	62	5	1240	Bormann and Gordon (1984)
Alnus rubra	146	5	10091	Bormann and Gordon (1984)
Alnus rubra	390	15–20		Binkley (1981)
Alnus rubra	30–55	2–4	5000–8000	Tripp et al. (1979)
Alnus rubra	117	7		Zavitkovski and Newton (1968)
Alnus rubra	244	30		Zavitkovski and Newton (1968)
Alnus viridis ssp. *sinuata*	130	5		Binkley (1982)
Alnus viridis ssp. *sinuata*	110	15–20		Binkley (1981)
Alnus viridis ssp. *viridis*	43			Moiroud and Capellano (1979)

Adapted from Binkley (1981) and Hurd et al. (2001)

estimates due to methodological difficulties (Aosaar et al. 2013). The spatial distribution patterns of nodules tend to be more homogeneous horizontally as a plantation ages (Rytter 1989) or with increasing tree size in a naturally established stand (Tobita et al. 2010a), although large variations in nodule distribution are also observed on the basis of stand age and tree size. These findings suggest that the distance from the *Alnus* tree must be considered to estimate nodule biomass in *Alnus* stands.

Seasonal variations in N_2 fixation activities and rates in *Alnus* stands

N_2 fixation begins shortly after leaf emergence in spring, remains high but variable in summer, decreases in late autumn, and ceases when all the leaves have been shed (Huss-Danell 1990; Tsutsumi et al. 1993; Sharma et al. 2010; Tobita et al. 2013a). Fluctuations in environmental conditions such as light, soil temperature, water, mineral nutrition, and pH as well as the presence of *Frankia* strains also affect nodule N_2 fixation activity (Pawlowski and Newton 2008; Gtari et al. 2013; Tobita et al. 2013b). Declines in N_2 fixation activity caused by these factors are often related to a deficiency of carbohydrates supplied from leaves to nodules because N_2 fixation activity depends on newly formed photosynthates supplied by the host plant (Huss-Danell 1997). Nonstructural carbohydrate accretion is greater in nodules in fall, contributing to the maintenance of overall plant levels of N_2 fixation similar to those observed during summer (Kaelke and Dawson 2005). Therefore, N_2-fixing root nodules are a strong metabolic sink for photosynthates within a plant (Huss-Danell and Sellstedt 1983; Ruess et al. 2006). This character of nodules may help *Alnus* species adapt to photosynthesis under elevated CO_2 conditions (Koike et al. 1997; Tobita et al. 2010b, 2011), as will be described in detail hereinafter.

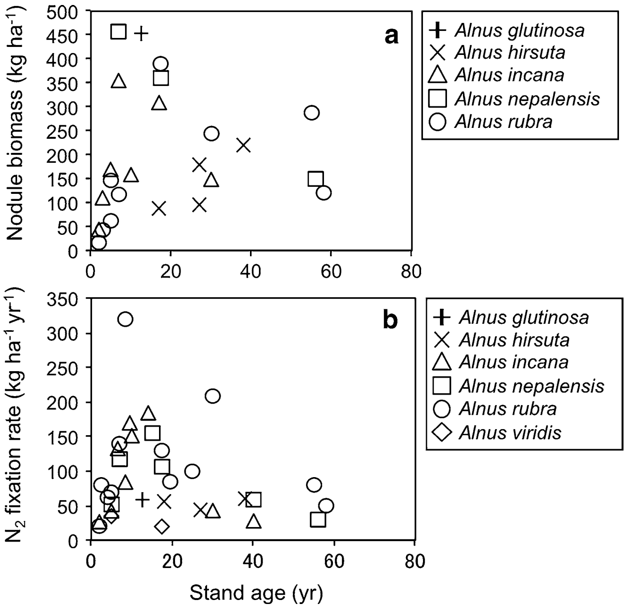

Fig. 1 Relationships between stand age and nodule biomass (**a**) and N_2 fixation rate (**b**) in *Alnus* stands. Adapted from Tables 1 and 2

Alnus species N_2 fixation rates are estimated to be several kg N ha^{-1} $year^{-1}$ to 320 kg N ha^{-1} $year^{-1}$ after several major assumptions are met (Table 2; e.g., Binkley 1981; Hibbs and Cromack 1990; Rytter et al. 1991; Cleveland et al. 1999; Hurd et al. 2001; Lõhmus et al. 2002; Uri et al. 2004; Lee and Son 2005). The nitrogenase activity of each nodule and N_2 fixation rate per plant vary depending on the N demand with increasing tree age (Son et al. 2007). The N_2 fixation rate increased with stand age and was higher in 10- to 20-year-old stands than in older stands (Fig. 1b). The contribution of N_2 fixation to N economy increases with stand age, peaking in a 15- to 20-year-old *A. nepalensis* stand (Sharma et al. 2002).

While many studies listed in Table 2 adopted an acetylene reduction assay (ARA) to evaluate the N_2 fixation activity, ARA results should be interpreted cautiously because ARA has been the subject of many criticisms due to assay inconsistency (Winship and Tjepkema 1990; Silvester et al. 2008). One is the so-called C_2H_2-induced decline in NA, which is apparent to varying degrees in actinorhizal nodules, including those of *Alnus* species (Tjepkema et al. 1988; Schwintzer and Tjepkema 1997). This decline is often followed by either partial or full recovery, which is dependent on the host species, growth conditions, and plant age (Silvester and Winship 1990). In addition, the conversion rate of C_2H_2 reduced to fixed N_2 in the ARA, which was set to 3:1 to compare the N_2 fixation data in Table 2, can also produce result errors (Winship and Tjepkema 1990). The actual ratio of acetylene reduction to N_2 fixation must be determined using ^{15}N-labeled dinitrogen concurrently (Schwintzer and Tjepkema 1997).

Facilitating effects of N_2 fixation in the *Alnus*–*Frankia* symbiotic relationship

N input into soil through N_2 fixation by *Alnus* species boosts N soil content (Wurtz 1995; Rhoades et al. 2001; Myrold and Huss-Danell 2003; Uri et al. 2014) and enhances the leaf N content and growth rates of mixed-planted trees (Vogel and Gower 1998; Brockley and Sanborn 2003; Roggy et al. 2004; Avendano-Yanez et al. 2014). However, some negative effects of N_2-fixing *Alnus* species have been reported such as the competition for light and soil nutrients (Chapin et al. 1994; Brockley and Sanborn 2003; Simard et al. 2006; Chapin et al. 2011), N leaching, gaseous N emissions due to denitrification (Compton et al. 2002; Mander et al. 2008, 2015), and the issue of invasion (Hiltbrunner et al. 2014).

Alnus species usually exhibit lower N resorption rates than those of non-N_2-fixers (Uliassi and Ruess 2002). These lower N_2 resorption rates can cause relatively high photosynthetic rates in autumn, which may help retain relatively high N_2 fixation activity (Tateno 2003; Tobita et al. 2013a). In addition, because a low N resorption rate will produce fallen leaves with higher N content, the *Alnus* species leaf litter decomposition rate is usually faster than that of other non-N_2-fixers (Sharma et al. 2008). The initial C/N ratio of leaf litter in non-N_2-fixers is usually higher, and their decomposition rate is slower than those of N_2-fixing species (Tateno et al. 2007). The leaf litter C/N ratio decreases rapidly from 20 to 12 in *A. hirsuta* (Tobita et al. 2013a) and from 20.5 to 15 in *A. japonica* (Yoon et al. 2014), suggesting that litter decomposition immediately moves into the mineralization stage (Takeda 1998). Another feature of *Alnus* species is the high susceptibility of leaves to herbivore damage (Kikuzawa et al. 1979; Tadaki et al. 1987; Tobita et al. 2013a), and their feces are a N input pathway to soils (Meehan and Lindroth 2007). In addition, symbiotic N_2 fixation in *Alnus* species may affect the distribution patterns of regenerated tree species (Tobita et al. 2015) and diversity (Hanley et al. 2006) as well as improve soil fertility. In early successional stages, *Alnus* species are used as nurse trees and may have a mothering role with these regenerated endozoochorous tree species.

Alnus species often regenerate easily during the early stages of succession (Bormann and Sidle 1990). However, it is occasionally difficult for *Alnus* species to recruit and expand their distribution in areas where *Frankia* densities are low (Seeds and Bishop 2009). The availability of infective *Frankia* and their compatibility with the host may limit the successful formation of root nodules capable of N_2 fixation (Markham and Chanway 1999). Symbiotic *Frankia* assemblages can differ widely between sympatric *Alnus* spp. and between successional habitats occupied by a given host species (Anderson et al. 2009). Phylogenetic

Table 2 Estimates of annual N_2 fixation in *Alnus* stands

Species	N_2 fixation (kgN ha^{-1} $year^{-1}$)	Stand age (years)	References	Methods[a]	Conversion rate[b]
Alnus glutinosa	58	5–20	Akkermans and van Dijk (1976)	AR	
Alnus hirsuta	56	18	Tobita et al. (2013a)	AR	3
Alnus hirsuta	41–47	27	Son et al. (2007)	AR	3
Alnus hirsuta	60	38	Lee and Son (2005)	AR	3
Alnus incana ssp. *incana*	152	10	Uri et al. (2011)	ACC	
Alnus incana ssp. *incana*	42	5	Uri et al. (2004)	ACC	
Alnus incana ssp. *incana*	185	14	Lõhmus et al. (2002)	ACC	
Alnus incana ssp. *incana*	28	40	Lõhmus et al. (2002)	ACC	
Alnus incana ssp. *incana*	27 (20)	2	Huss-Danell et al. (1992)	AR	3 (4)
Alnus incana ssp. *incana*	113–153 (85–115)	6–7	Rytter et al. (1991)	AR	3 (4)
Alnus incana ssp. *incana*	43 (32)	30	Johnsrud (1978)	AR	3 (4)
Alnus incana ssp. *rugosa*	49 (37)		Hurd et al. (2001)	AR	3 (4)
Alnus incana ssp. *rugosa*	1–5 (1–4)		Younger and Kapustka (1983)	AR	3 (4)
Alnus incana ssp. *rugosa*	85	1–16	Voigt and Steucek (1969)	ACC	
Alnus incana ssp. *rugosa*	170	1–18	Daly (1966)	ACC	
Alnus nepalensis	57–155	15–20	Sharma et al. (2010)	AR	3
Alnus nepalensis	52	5	Sharma et al. (2002)	AR	3
Alnus nepalensis	155	15	Sharma et al. (2002)	AR	3
Alnus nepalensis	59	40	Sharma et al. (2002)	AR	3
Alnus nepalensis	117	7	Sharma and Ambasht (1988)	AR	3
Alnus nepalensis	29	56	Sharma and Ambasht (1988)	AR	3
Alnus rubra	140	7	Zavitkovski and Newton (1968)	GAN	
Alnus rubra	209	30	Zavitkovski and Newton (1968)	GAN	
Alnus rubra	62	4	Tripp et al. (1979)	AR	3
Alnus rubra	75–85	55	Binkley et al. (1992)	AR	3
Alnus rubra	18–23	2	Kim (1987)	AR	3
Alnus rubra	50	58	Kim (1987)	AR	3
Alnus rubra	70	5	Bormann and Gordon (1984)	AR	3
Alnus rubra	130	15–20	Binkley (1981)	AR	3
Alnus rubra	100	10–40	Bormann and DeBell (1981)	ACC	
Alnus rubra	80	1–4	DeBell and Radwan (1979)	ACC	
Alnus rubra	85	1–38	Cole et al. (1978)	ACC	
Alnus rubra	320	2–15	Newton et al. (1968)	ACC	
Alnus tenuifolia	59		Uliassi and Ruess (2002)	AR	3
Alnus viridis ssp. *sinuata*	10–15		Sanborn et al. (2002)	15N	
Alnus viridis ssp. *sinuata*	35	5	Binkley (1982)	AR	3
Alnus viridis ssp. *sinuata*	20 (15)	15–20	Binkley (1981)	AR	3 (4)
Alnus viridis ssp. *viridis*	8 (6)		Moiroud and Capellano (1979)	AR	3 (4)

Adapted from Binkley (1981), Hibbs and Cromack (1990), and Hurd et al. (2001)

[a] N_2 fixation estimate method; *AR* acetylene reduction assay; *ACC* accretion study; *GAN* greenhouse accretion per gram of nodule × nodule biomass in the field; *15N* ^{15}N isotope dilution method

[b] Molar conversion ratio of acetylene reduction to N_2 fixation

specificity is a significant factor in the *Alnus tenuifolia*–*Frankia* interaction, and significant habitat-based differentiation may exist among *A. tenuifolia*-infective genotypes (Anderson et al. 2013). The global biogeographic community of *Alnus*-associated *Frankia* (Benson and Dawson 2007; Põlme et al. 2014) and the genetic diversity of *Frankia* populations in the soil and root nodules (Pokharel et al. 2011) have also been evaluated.

Predicted effects of elevated CO_2 on *Alnus–Frankia* N_2 fixation

Increasing CO_2 is an important factor influencing global climate change (IPCC 2013) and nutrient availability, particularly that of soil N, may strongly limit the growth response of woody plants to elevated CO_2 (Norby et al. 2010; Zak et al. 2011; Sigurdsson et al. 2013) because forest ecosystems are usually N limited (Wang et al. 2010). Symbiotic N_2 fixation may play an important role as exogenous N input to sustain the enhanced growth of non-N_2-fixers under an elevated CO_2 environment (Finzi et al. 2007). However, because N_2 fixation is influenced by several abiotic and biotic factors, it is predicted that N_2-fixers, such as *Alnus* species, do not always enhance their N_2 fixation ability under elevated CO_2 (Tobita et al. 2010b). We will discuss the probable responses of *Alnus* species to elevated CO_2, considering other factors, such as N, P, and water conditions, by reviewing the results from chamber experiments (Tobita et al. 2011) and recent free-air CO_2 enrichment (FACE) experiments (Millett et al. 2012). In addition, we will review the understanding of the effects of elevated ozone (O_3) (Wittig et al. 2009) and leaf chemistry in relation to herbivores (Koike et al. 2006), which can decrease the growth of *Alnus* species.

Photosynthetic and growth responses to elevated CO_2 in *Alnus* species

As N_2 fixers in legumes and actinorhizal plants are largely independent of soil N content, they may respond to elevated CO_2 more directly than non-N_2-fixers by increasing the photosynthetic and growth rates (Temperton et al. 2003a; Reverchon et al. 2012). Excellent reviews have summarized these responses in legumes (Ainsworth and Long 2004; Ainsworth and Rogers 2007; Rogers et al. 2009). *Alnus* species exhibit a photosynthetic acclimation response to elevated CO_2 (Vogel and Curtis 1995), which means they increase their photosynthetic rates under elevated CO_2 compared to those under ambient CO_2 even in N_2-deficient soil (Koike et al. 1997; Tobita et al. 2010b, 2011), rather than downregulate photosynthesis (Long et al. 2004; Ainsworth and Rogers 2007). *Alnus hirsuta* saplings used in FACE experiments in Japan also did not downregulate photosynthesis in infertile soil, whereas photosynthesis was downregulated in two *Betula* species under elevated CO_2, regardless of the soil fertility (Eguchi et al. 2008a). As mentioned in the previous section, the N_2-fixing root nodules of *Alnus* species act as a strong metabolic sink for photosynthates to avoid photosynthetic downregulation under elevated CO_2 conditions. Biomass production by *Alnus* species is significantly stimulated by increasing CO_2 in the presence of *Frankia* species, whereas they show no response to elevated CO_2 in the absence of *Frankia* species (Pourhassan et al. 2015).

Interactive effects of soil N and elevated CO_2 on N_2 fixation in *Alnus* species

The positive photosynthetic response to elevated CO_2 by N_2-fixing plants increases the C supply to root nodules (Tissue et al. 1997), which may stimulate N_2 fixation in trees. N_2 fixation may be adjusted in response to environmental change, either through variations in nodule biomass or NA (Valverde et al. 2002). Elevated CO_2 increases the total amount of N_2 fixed per *Alnus* species plant because of increased nodule mass (Hibbs et al. 1995; Tobita et al. 2010b) and NA (Temperton et al. 2003a), or both (Norby 1987; Arnone and Gordon 1990; Vogel et al. 1997), as reported by several growth chamber and open-top chamber experiments. One important level of plant control during actinorhizal symbiosis may be the regulation of the proportion of symbiotic tissue in the plant relative to plant biomass allocation (Wall and Berry 2008). Moreover, elevated CO_2 has no effect on the relationship between plant mass and nodule mass, even when nodule biomass increases under elevated CO_2 conditions (Hibbs et al. 1995; Tobita et al. 2005, 2010b). These results suggest that elevated CO_2 enhances nodule mass as a function of the increasing total plant mass, rather than by enhancing the allocation of biomass to roots and nodules.

Soil mineral N content often limits nodule formation and NA because larger quantities of photosynthates are needed for N_2 fixation compared with N, which can be absorbed from the soil (Ekblad and Huss-Danell 1995; Vogel et al. 1997; Lambers et al. 2008; Wall and Berry 2008; Chapin et al. 2011). Thomas et al. (2000) suggested that elevated CO_2 mitigates these inhibitory effects of substrate N in leguminous tree species, either through increased allocation of C to nodules or through increased N demand by the plant. However, increased soil N availability has a negative effect on nodule production and biomass allocation to nodules in *Alnus* species, regardless of CO_2 treatment (Koike et al. 1997; Bucher et al. 1998; Temperton et al. 2003b; Tobita et al. 2005). These results indicate that the inhibitory effect of high soil N availability on nodulation in *Alnus* species is retained even under elevated CO_2 levels.

N_2 fixation response in *Alnus* species subjected to FACE experiments

Only two FACE experiments have been reported on *Alnus* species. One was conducted in Japan (Hokkaido), as introduced in the previous section, on the responses of *A. hirsuta* to elevated CO_2 in fertile and infertile soils compared to those of non-N_2-fixing deciduous tree species, including *Betula platyphylla*, *Betula maximowicziana*, *Quercus mongolica*, and *Fagus crenata* (Agari et al. 2007; Eguchi et al. 2008a, 2008b; Watanabe et al. 2010). The other was the UK Bangor FACE experiment in which the effects of elevated CO_2 on *A. glutinosa* performance were compared between monocultures and mixed plantings of *Betula pendula, Fagus sylvatica*, and *Populus tremula* × *tremuloides* (Hoosbeek et al. 2011; Millett et al. 2012; Smith et al. 2013a, 2013b; Godbold et al. 2014; Scullion et al. 2014). N_2 fixation in *A. glutinosa* increases under elevated CO_2 despite the absence of significant growth stimulation in a mixed-species stand after 4 years (Millett et al. 2012). However, the fraction of N_2 derived from N_2 fixation, calculated using the ^{15}N natural abundance method (Chaia and Myrold 2010; Zhang et al. 2014), was unaffected by the elevated CO_2 in an *A. glutinosa* monoculture stand, indicating no increase in N_2 fixation under elevated CO_2 in a monoculture although plant biomass increased significantly (Hoosbeek et al. 2011). These differences in responses to elevated CO_2 may be related to enhanced growth rate, N uptake, and N_2 fixation of *A. glutinosa* in a mixed stand compared to those in a monoculture due to increased ecosystem resource utilization through belowground niche differentiation among trees (Smith et al. 2013a). In contrast, N_2 fixation in *Lupinus* species legumes increases under elevated CO_2 in both a monoculture and a mixed grassland system in a FACE experiment (Lee et al. 2003). Plants rarely grow in isolation, and their response to elevated CO_2 can be affected by the extent and type of plant–plant interactions (Poorter and Navas 2003). Understanding how mixed-species forests respond to elevated CO_2 will be essential to assess forest growth dynamics including the response of N_2 fixation in *Alnus* species and improving the parameterization of global change cycle models (Norby and Zak 2011).

N allocation in *Alnus* species under elevated CO_2

N concentrations generally decline in plant tissues under elevated CO_2 (Ainsworth and Rogers 2007; Sardans and Peñuelas 2012). N uptake is not affected as much as C uptake, whereas increased CO_2 alters the plant C/N balance (Kallarackal and Roby 2012). The increase in the total *Alnus* species plant N mass under elevated CO_2 is smaller than that predicted by the response of the total biomass to elevated CO_2 in phytotron experiments (Temperton et al. 2003b; Tobita et al. 2011). The same phenomenon was observed in FACE studies, which showed increased N use efficiency in an *A. glutinosa* monoculture stand under elevated CO_2 (Millett et al. 2012; Pourhassan et al. 2015). These results suggest that it is necessary to evaluate biomass accumulation as well as total N content and its allocation when considering the N_2-fixing ability of *Alnus* species under elevated CO_2.

Interactive effects of soil P and elevated CO_2 on N_2 fixation in *Alnus* species

N availability limits plant responses to elevated CO_2 (Norby et al. 2010). However, higher soil N availability under elevated CO_2 does not necessarily lead to higher plant biomass production (Körner et al. 2005; Schleppi et al. 2012) because stoichiometric constraints extend to elements other than N, such as P, or some micronutrients. More generally, any biomass response to elevated CO_2 is controlled by the stoichiometric balance among many elements required to construct new tissues and used for active metabolism (Hungate et al. 2004; Sardans and Peñuelas 2012; Leuzinger and Härrenschwiler 2013). Many studies have focused on N but P limitations are also common in many terrestrial ecosystems (Nord and Lynch 2009; Wang et al. 2010). In addition, P is unlikely to increase in the future because it is a non-renewable resource (Pandey et al. 2015). N_2 fixation in actinorhizal plants as well as legumes is a P-consuming activity that accompanies the synthesis of DNA and plasma membranes for cell division during nodule development and ATP synthesis to reduce N (Gentili et al. 2006). Therefore, P is often the most growth-limiting nutrient for actinorhizal plants because of the relatively high demand for P compared to that of non-N_2-fixers (Ingestad 1981; Uliassi et al. 2000; Brown et al. 2011). P deficiency limits nodule formation and N_2 fixation in *A. incana*, even under ambient CO_2 conditions (Gentili and Huss-Danell 2003; Ruess et al. 2013). Although studies on the combined effects of elevated CO_2 and P deficiency on N_2 fixation are scarce, N_2 fixation per plant in two *Alnus* species does not increase under elevated CO_2 and P-deficient conditions because plant growth is strongly suppressed and nodule formation is inhibited without a marked change in NA (Tobita et al. 2010b). In the BangorFACE experiment, Smith et al. (2013a) suggested that soil P availability, rather than N, have been a limiting factor compared to that at other FACE sites because the experimental site was established on former agricultural soils. Legumes exhibit a particularly strong increase in the biomass production under combined elevated CO_2 and P fertilization conditions in grassland communities (Stöcklin and Körner 1999), indicating a stoichiometric control of the CO_2 effects (Leuzinger and Hättenschwiler 2013). The increased demand for P to support an increased growth rate may be another key constraint on plant responses to elevated CO_2 (Kogawara et al. 2006). Tripartite symbiosis with mycorrhiza (Yamanaka et al. 2003; Urgiles et al. 2014) may play an important role to meet the increased P demand and to effectively utilize P under projected elevated CO_2 conditions.

Interactive effects of drought and elevated CO_2 on N_2 fixation in *Alnus* species

Increasing temperatures have been predicted to decrease precipitation during summer (Calfapietra et al. 2010). Drought conditions have the potential to decrease the photosynthetic rates and limit growth through stomatal closure (Flexas and Medrano 2002), which may also affect symbiotic N_2 fixation. Drought limits the N_2-fixing capacity by limiting metabolic capacity of bacteria and by generating oxidative damage in legumes (Aranjuelo et al. 2011). Several *Alnus* species such as *A. rubra* are sensitive to water stress (Pezeshki and Hinckley 1988), and NA is sensitive to short-term drought (Huss-Danell 1997). In contrast, NA in *A. glutinosa* shows no marked decrease after adaptation to moderately dry soil (Seiler and Johnson 1984). Most studies that have been conducted on elevated CO_2 and drought indicate that elevated CO_2 tends to ameliorate the negative effects of drought on the net photosynthetic rates and biomass accumulation (Kitao et al. 2007; Sicher and Barnaby 2012; Feng et al. 2014). Plant growth is stimulated by elevated CO_2 in C_3 species, even under moderate drought conditions (Xu et al. 2013). The light-saturated net photosynthetic rate and growth of *A. hirsuta* and *A. maximowiczii* are also enhanced by elevated CO_2 under sufficient P conditions, even in dry soil (Tobita et al. 2010b). However, when soil P is limiting, these two *Alnus* species have no positive responses to elevated CO_2. Elevated CO_2 alters leaf water potential of these two *Alnus* species in wet soil (Uemura et al. 2009) and increases the susceptibility to photoinhibition (Tobita et al. 2008). These results suggest that the risk of an occasional severe drought increases under elevated CO_2, particularly when these *Alnus* species are grown in wet soil.

Sensitivity to tropospheric ozone under elevated CO_2

Tropospheric O_3 levels have increased globally since preindustrial times (IPCC 2007, 2013) and continue to rise, particularly in East Asia (Fowler et al. 2008). Ozone and CO_2 are two major anthropogenic air pollutants with opposing impacts on plant growth (Lindroth 2010; Leisner and Ainsworth 2012) because increased O_3 reduces net photosynthesis. *Alnus* species are relatively sensitive to O_3, and *A. incana* have been used as O_3 bioindicators in Europe (Manning et al. 2002; Manning and Godzik 2004). Surface ambient background O_3 over land in the northern hemisphere has already increased to levels that have decreased growth in several tree species (Matyssek et al. 2007), including *Alnus viridis* (VanderHeyden et al. 2001). In addition, increased O_3 decreases shoot and root dry weights and enhances leaf senescence in *A. incana* (Mortensen and Skre 1990; Wittig et al. 2009). Although the enhanced growth of *Alnus* species will be expected to increase the N input in ecosystems under future elevated CO_2 conditions, the projected increase in future O_3 level may decrease the growth of *Alnus* species.

The legume soybean is an O_3 sensitive crop (Mills et al. 2007). Rising O_3 decreases yield (Morgan et al. 2003; Long et al. 2005) and alters the gene expression in the reproductive tissues of soybean (Leisner et al. 2014). Root biomass and the number of root nodules decreased in two clover species in response to O_3, and one revealed a reduced N_2 fixation rate under elevated O_3 (Hewitt et al. 2014). In contrast, intact subalpine grassland communities that include legumes (clover) show low sensitivity to O_3, despite the high O_3 sensitivity found in earlier experiments using pot-grown plants (Bassin et al. 2013).

Considering the interactive effects of elevated CO_2 and O_3, a key question is whether elevated CO_2 will ameliorate the negative effect of O_3, which is an oxidative stressor in plants, or whether O_3 will offset the positive effect of elevated CO_2 on plant growth (Feng et al. 2014). A FACE experiment on soybean (SoyFACE) was performed to investigate the interactive effects of elevated CO_2 and O_3 (Gillespie et al. 2012) and revealed that growth under elevated CO_2 conditions could decrease many of the negative effects of elevated O_3 on plant physiology. However, a clover FACE experiment in the forest understory (AspenFACE) showed that enriched CO_2 and O_3 have large direct and indirect effects on colonization, establishment, and performance (Awmack et al. 2007). Thus, it will become important to also determine the interactive effects of elevated CO_2 and O_3 on N_2 fixation by *Alnus* species to predict N supply in future forest ecosystems.

Effect of elevated CO_2 on *Alnus* species leaf chemistry

Elevated CO_2 and O_3 can change leaf chemistry, such as C, N, P, lignin, and secondary metabolites (Lindroth 2012). In addition, these changes in leaves can alter leaf litter quality, which may affect palatability to detritivores, decomposition, and nutrient turnover (Dray et al. 2014). The defense capacity of broadleaf trees usually increases under elevated CO_2 (Lindroth 2010, 2012). However, the survival rates and longevity of silkworm fed *A. hirsuta* leaves are independent of CO_2 level, unlike what occurs with the non-N_2-fixers *Betula platyphylla*, *Quercus mongolica*, and *Acer* mono. In addition, the survival rates and longevity of silkworms are enhanced by infertile soil (Koike et al. 2006). *Alnus hirsuta* leaves do not have increased levels of

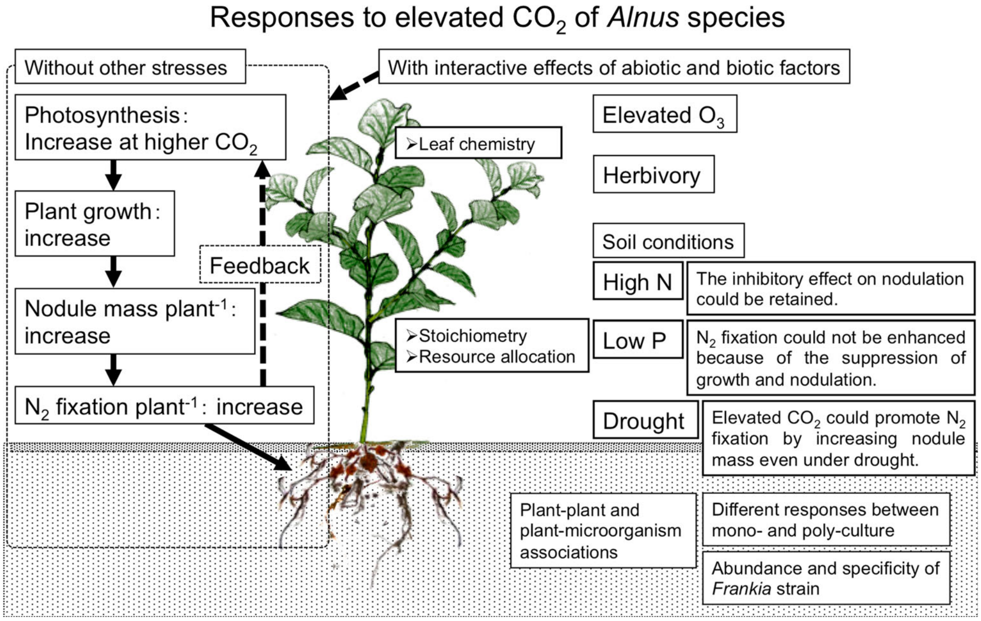

Fig. 2 Schematic diagram of the responses of growth and N_2 fixation in *Alnus* species to elevated CO_2 with and without the interactive effects of many abiotic and biotic factors

defense chemicals in plants held in an elevated CO_2 environment (Koike et al. 2006; Agari et al. 2007), whereas the concentrations of condensed tannins in the leaves of *A. maximowiczii*, which show limited leaf production than that of *A. hirsuta*, are much higher than those of *A. hirsuta*, even under ambient CO_2 and increased under elevated CO_2 (Agari et al. 2007). These results indicate that some variations in the defense strategy may occur under elevated CO_2 conditions, even within *Alnus* species. The litter chemistry of *A. glutinosa* was largely unaffected by elevated CO_2 in a FACE experiment, unlike that of *Betula pendula* (Dray et al. 2014). The feeding behavior of invertebrates on *Alnus* leaves shows large species-specific variations (Dray et al. 2014; Scullion et al. 2014), and only two invertebrate species revealed compensatory feeding when consuming more elevated-CO_2 litter than litter produced under ambient-CO_2. A few studies have evaluated the impacts of elevated O_3 on soil invertebrate performance and litter decomposition (Lindroth 2012). Therefore, it is necessary to conduct multi-factorial FACE experiments under both elevated CO_2 and O_3 using *Alnus* species (Kawaguchi et al. 2012; Lindroth 2012; Kostiainen et al. 2014) to better understand whether N_2 fixation by *Alnus* species is a potential source of N in forest ecosystem under elevated CO_2 conditions.

Conclusion

The *Alnus–Frankia* symbiotic relationship fixes as much N as that of the legume–*Rhizobium* symbiotic relationship and has been utilized to revegetate and rehabilitate N-deficient disturbed areas. In addition, N_2 fixation by *Alnus* species may affect the distribution pattern of regenerated plants while improving soil fertility. N_2 fixation because of *Alnus–Frankia* symbiosis could supply an important source of N needed to sustain increased N uptake due to high rates of forest productivity in the face of global climate change under elevated CO_2. However, recent findings including those from FACE experiments, suggest that the response of N_2 fixation to elevated CO_2 in *Alnus* species depends on the composition of mixed non-N_2-fixing species and that soil N and P availability as well as many other abiotic and biotic factors also have interactive effects on N_2 fixation (Fig. 2). Because elevated CO_2 can alter plant N and P contents and stoichiometry, it will be necessary to evaluate N mass allocation as well as biomass accumulation when investigating the N_2 fixing ability of *Alnus* species. In addition, because *Alnus* species are relatively sensitive to O_3, determining the responses of *Alnus* species to increased CO_2 and O_3 levels will be important to predict N supply in future forest ecosystems.

Author contribution statement H. Tobita wrote the manuscript. H. Tobita, K. Yazaki, H. Harayama, and M. Kitao compiled the review. K. Yazaki, H. Harayama, and M. Kitao revised the manuscript.

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Compliance with ethical standards

Conflict of interest We have no conflict of interest.

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