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Abstract: Methodologies based on 15N enrichment (E) and 15N natural abundance (NA) have been used to obtain quantitative estimates of the response of biological N2 fixation (BNF) of legumes (woody, grain and forage) and actinorhizal plants grown in artificial media or in soil exposed to elevated atmospheric concentrations of carbon dioxide e[CO2] for extended periods of time, in growth rooms, greenhouses, open top chambers or free-air CO2 enrichment (FACE) facilities. 15N2 has also been used to quantify the response of endophytic and free-living diazotrophs to e[CO2]. The primary criterion of response was the proportional dependence of the N2-fixing system on the atmosphere as a source of N. i.e. the symbiotic dependence (Patm). The unique feature of 15N-based methods is their ability to provide time-integrated and yield-independent estimates of Patm. In studies conducted in artificial media or in soil using the E methodology there was either no response or a positive response of Patm to e[CO2]. The interpretation of results obtained in artificial media or with 15N2 is straight forward, not being subject to the assumptions on which the E and NA soil-cultured methods are based. A variety of methods have been used to estimate isotopic fractionation attendant on the NA technique, the so-called 'B value', which attaches a degree of uncertainty to the results obtained. Using the NA technique, a suite of responses of Patm to e[CO2] has been published, from positive to neutral to sometimes negative effects. Several factors which interact with the response of N2-fixing species to e[CO2] were identified.

Response to Reviewers: Please see attached responses to reviewers' comments.

Highlights (Revised)

- A critical review of ^{15}N methods to quantify CO_2 effects on N_2 fixation is provided
- $^{15}N_2$ and ^{15}N dilution (enrichment or natural abundance) techniques were reviewed
- ^{15}N estimates of symbiotic dependence are time-integrated and yield-independent
- 26 papers covering woody and herbaceous N_2-fixing plants were evaluated
- Inconsistent responses to elevated [CO_2] were identified

Title page

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^{15}N methodologies for quantifying the response of N_2-fixing associations to elevated $[CO_2]$: A review

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ABSTRACT

Methodologies based on ^{15}N enrichment (*E*) and ^{15}N natural abundance (*NA*) have been used to obtain quantitative estimates of the response of biological N_2 fixation (BNF) of legumes (woody, grain and forage) and actinorhizal plants grown in artificial media or in soil exposed to elevated atmospheric concentrations of carbon dioxide e[CO_2] for extended periods of time, in growth rooms, greenhouses, open top chambers or free-air CO_2 enrichment (FACE) facilities. $^{15}N_2$ has also been used to quantify the response of endophytic and free-living diazotrophs to e[CO_2]. The primary criterion of response was the proportional dependence of the N_2-fixing system on the atmosphere as a source of N. i.e. the symbiotic dependence (P_{atm}). The unique feature of ^{15}N-based methods is their ability to provide time-integrated and yield-independent estimates of P_{atm}. In studies conducted in artificial media or in soil using the *E* methodology there was either no response or a positive response of P_{atm} to e[CO_2]. The interpretation of results obtained in artificial media or with $^{15}N_2$ is straight forward, not being subject to the assumptions on which the *E* and *NA* soil-cultured methods are based. A variety of methods have been used to estimate isotopic fractionation attendant on the *NA* technique, the so-called 'B value', which attaches a degree of uncertainty to the results obtained. Using the *NA* technique, a suite of responses of P_{atm} to e[CO_2] has been published, from positive to neutral to sometimes negative effects. Several factors which interact with the response of N_2-fixing species to e[CO_2] were identified.

Keywords: ^{15}N; $\delta^{15}N$; elevated [CO_2]; legumes; symbiotic dependence; stable isotope tracer

1. Introduction

The response of components of terrestrial ecosystems to increasing concentrations of atmospheric carbon dioxide [CO_2] has been a scientific line of enquiry since the 1960s. Initially, growth chamber experiments were conducted but were then followed by open top chambers and free-air CO_2 enrichment (FACE) facilities. Contributions of biological N_2 fixation (BNF) via free-living, endophytic or symbiotic micro-organisms are collectively an important source of N addition to natural and agricultural ecosystems. It is therefore important to understand if BNF may be affected by increasing atmospheric [CO_2], and in which way and by how much. Early studies relied on the use of the acetylene reduction assay or other biochemical markers to obtain qualitative estimates of the response of BNF to [CO_2], but such methods are clearly inadequate and quantitative estimates are needed.

The quantitative estimation of the proportional contribution of N_2 fixation to the N nutrition of soil-cultured legumes or other N_2-fixing associations (i.e. the symbiotic dependence, P_{atm}) is accomplished by the application of ^{15}N methodologies which are either direct ($^{15}N_2$) or indirect (^{15}N dilution), the latter generally being based either on artificial ^{15}N enrichment or ^{15}N natural abundance (Chalk, 2016; Chalk et al., 2016). For the estimation of the amount of N_2 fixed (i.e. the symbiotic performance), the N yield (i.e. dry matter yield × N concentration) is multiplied by P_{atm}. In order to separate the effect of any given variable on symbiotic performance, both N yield and P_{atm} must be determined independently, which is the unique strength of the ^{15}N methodologies. Several studies have demonstrated that P_{atm} is more resilient to stress factors such as nutrient deficiencies (Chalk, 2000), sodicity (Smith et al., 2009) or drought (Chalk et al., 2010) compared to N yield, and that the stress must be acute before P_{atm} is significantly reduced.

A general review of the literature on the effect of elevated [CO_2] (e[CO_2]) on legume BNF was provided by Rogers et al. (2009). A meta-analysis of published data on estimates of the effect of e[CO_2] on P_{atm} and N yield of grain and pasture legumes was published by Lam et al. (2012 b). From a data set of 27 observations from 9 studies it was concluded that there was a 38% increase in the yield of fixed N and a 10% (non-significant) increase in P_{atm} due to e[CO_2] from a range of 550 to 730 μmol mol^{-1}, indicating that P_{atm} was much less responsive compared with N yield. The objective of the present review is to revisit this subject by casting a wider net for published ^{15}N-based yield-independent data on legume response to e[CO_2], including not only grain and forage legumes, but also woody legumes, and to extend the coverage further to include actinorhizal, endophytic and free-living associations. Attention will be focused on the correct applications of ^{15}N-based technologies. The aim is to gain an overall quantitative assessment of the effect of e[CO_2] on symbiotic dependence and symbiotic performance and to consider a range of factors which may play an interacting role. We shall not attempt to provide an assessment of the physiological basis for the observed responses or lack thereof.

2. The quantitative response of N_2-fixing plants to e[CO_2]: ^{15}N methodologies

2.1. CO_2 enrichment techniques

The three most widely used CO_2 enrichment techniques are glasshouses/growth chambers, open top chambers (OTC) and free-air CO_2 enrichment (FACE) facilities. While economically practical for a [CO_2] controlled environment, growth chambers are generally limited in scale to accommodate pots or soil cores. Open top chambers (OTC) can house larger scale experiments in the field, but natural wind flow is prevented and the microenvironment is altered by the chamber. Free-air CO_2 enrichment (FACE) facilities are generally favored for larger scale field studies, and although expensive to operate continually there is no perturbation of microclimate within. Several reviews have been written about [CO_2] enrichment facilities, one of the most recent being Uprety et al. (2006). The concentration of CO_2 [CO_2] has been expressed in several units. The standard unit is μmol mol^{-1} which is equivalent to μl l^{-1} or ppm. If expressed as a partial pressure (pCO_2) in Pa, then 1 Pa = 10 μbar = 9.869 μmol mol^{-1}. Henceforth, a[CO_2] will denote the ambient concentration of CO_2 while e[CO_2] will denote the elevated concentration of CO_2.

2.2. Literature search

To assess the effect of e[CO_2] on BNF, we performed extensive keyword searches of several databases (Web of Science, Scopus, CAB Abstracts, Academic Search complete and Google Scholar) for studies published prior to March 2016. The keywords used in the search included elevated CO_2, (biological) N_2 fixation, ^{15}N, ^{15}N natural abundance, ^{15}N dilution, ^{15}N enrichment, legumes, and their combinations. The search resulted in 26 studies (Tables 1–6).

2.3. Plant culture in artificial media

2.3.1. ^{15}N enrichment (E)

The effect of e[CO_2] on N_2 fixation by plant-microbial associations has been studied by growing the plant under partially-controlled environmental conditions in an artificial rooting medium such as hydroponics or sand watered with ^{15}N-enriched nutrient solution. In this case P_{atm} is estimated according to Eq. 1.

$$P_{\mathrm{atm}} = 1 - \frac{E_{\mathrm{plant}}}{E_{\mathrm{solution}}} \tag{1}$$

Where E is the excess atom fraction ^{15}N. E_{plant} is the difference in the ^{15}N abundance (atom fraction ^{15}N) of the plant in the ^{15}N-enriched treatment minus the ^{15}N abundance of the plant in a control (*NA*) treatment. E_{solution} is the ^{15}N abundance of the solution minus the ^{15}N natural abundance of air (0.003 663 atom fraction ^{15}N).

The value of P_{atm} calculated according to Eq. 1 was adjusted by Zanetti et al. (1998) to compensate for the N yield of the plants at age 6 weeks (t_0) before the imposition of the e[CO_2] treatment for a further 36 days (t_1). The adjusted P_{atm} (Eq. 2) is thus yield dependent.

$$P_{\mathrm{atm}}(\mathrm{adjusted}) = \frac{\left(\mathrm{N\ yield}_{t_1} \times P_{\mathrm{atm}}\right) - \mathrm{N\ yield}_{t_0}}{\mathrm{N\ yield}_{t_1} - \mathrm{N\ yield}_{t_0}} \tag{2}$$

2.3.2. ^{15}N natural abundance (NA)

The artificial rooting medium may also contain plant-available N close to ^{15}N natural abundance. In this case, the N_2-fixing plant is grown in the solution, but in addition it must be grown in an N-free solution where the plant is wholly dependent on N_2 fixation, in order to determine the 'B value'. B is the isotopic fractionation associated with the process of BNF and its assimilation by the plant. In this case P_{atm} is estimated according to Eq. 3.

$$P_{\mathrm{atm}} = \frac{\delta^{15}\mathrm{N}_{\mathrm{solution}} - \delta^{15}\mathrm{N}_{\mathrm{plant}}}{\delta^{15}\mathrm{N}_{\mathrm{solution}} - \mathrm{B}} \qquad (3)$$

where δ^{15}N is the $\frac{^{15}\mathrm{N}}{^{14}\mathrm{N}}$ratio of the sample relative to the $\frac{^{15}\mathrm{N}}{^{14}\mathrm{N}}$ ratio of the international standard, atmospheric N_2 (Eq. 4).

$$\delta^{15}\mathrm{N} = \frac{\frac{^{15}\mathrm{N}}{^{14}\mathrm{N}}\mathrm{sample}}{\frac{^{15}\mathrm{N}}{^{14}\mathrm{N}}\mathrm{standard}} - 1 \qquad (4)$$

where by definition, $\delta^{15}\mathrm{N}_{\mathrm{standard}}$ is zero excess atom fraction ^{15}N.

2.4. Soil cultured plants

The symbiotic dependence (P_{atm}) of a soil-cultured N_2 fixing plant-microbial association can be determined by the direct $^{15}N_2$ technique, or indirect methods based on ^{15}N enrichment (E) or ^{15}N natural abundance (*NA*).

2.4.1. $^{15}N_2$

The direct method involves exposure of the whole plant or plant roots in an enclosure to N_2 highly enriched in ^{15}N (usually > 0.95 atom fraction ^{15}N) for variable time periods. However, because of problems such as the cost of the isotope, enclosure leakage and the need to maintain environmental conditions for normal plant growth ([CO_2], [O_2], humidity, temperature, light intensity), short duration times are the norm. Due to the above complexities, the method has seldom been used to study the effect of e[CO_2] on N_2 fixation (e.g. Dakora and Drake, 2000). P_{atm} is estimated according to Eq. 5.

$$P_{\mathrm{atm}} = \frac{E_{\mathrm{plant}}}{E_{\mathrm{atmosphere}}} \qquad (5)$$

where E_{plant} is the excess atom fraction ^{15}N of the plant and $E_{\mathrm{atmosphere}}$ is the excess atom fraction ^{15}N of the isotopically-enriched atmosphere. E_{plant} is determined as the difference in the ^{15}N abundance (atom fraction ^{15}N) of the plant exposed to $^{15}N_2$ minus the ^{15}N abundance of the same plant grown in a normal atmosphere. $E_{\mathrm{atmosphere}}$ is determined as the difference in the ^{15}N abundance (atom fraction ^{15}N) of the $^{15}N_2$ atmosphere minus the^{15}N natural abundance of air (0.003 663 atom fraction ^{15}N). In the absence of a plant, free-living heterotrophic N_2 fixation is estimated according to Eq. 5, but with E_{soil} replacing E_{plant} (Garten et al., 2008).

2.4.2. ^{15}N enrichment (E)

The indirect *E* method, as originally conceived, required the use of a non-N_2-fixing reference plant. P_{atm} is estimated by Eq. 6.

$$P_{\mathrm{atm}} = 1 - \frac{E_{\mathrm{N2-fixing\ plant}}}{E_{\mathrm{reference\ plant}}} \qquad (6)$$

Where *E* is the excess atom fraction ^{15}N. E_{plant} is the difference in the ^{15}N abundance (atom fraction ^{15}N) of the plant in the ^{15}N-enriched treatment minus the ^{15}N abundance of the plant in a control (unenriched) treatment.

Although the principles and assumptions on which the *E* method is based were articulated in an in-depth review by Chalk and Ladha (1999), misunderstandings and errors persist in the literature. We provide two examples of conceptual errors and one example of shortcomings in the way the *E* methodology was employed in studies of the effect of e[CO_2] on P_{atm}.

In the first example, Niklaus et al. (1998) and Niklaus and Köner (2004) claimed to have used a variation (Eq. 7) of the traditional formula (Eq. 6) for estimating P_{atm}.

$$P_{\mathrm{atm}} = \frac{A_{\mathrm{legume}} - A_{\mathrm{reference\ plant}}}{A_{\mathrm{reference\ plant}} - 0.003663} \qquad (7)$$

where *A* is ^{15}N abundance (atom fraction ^{15}N). Eq. 7 is in fact erroneous as it will give a negative estimate of P_{atm} since the ^{15}N abundance of the legume will be less than the ^{15}N abundance of the reference plant due to dilution of the labelled N taken up from the soil with unlabelled biologically-fixed N_2. Since the authors provided estimates of P_{atm} within the realm of possibility (88 ± 3 to 100 ± 2 %) one must conclude that a formula different from Eq. 7 was used.

A second example is the incorrect interpretation that the isotope dilution approach over-estimates P_{atm} because it does not account for N derived from the soil (Butterly et al., 2016). The assumption on which the *E* method is based is that the fixing and reference plants take up the *same proportions* of labelled (introduced isotope) and unlabelled (indigenous mineral N) from the soil N pool. In fact *different amounts* of N may be taken up by fixing and reference species as removal such as plant uptake does not in itself alter the ^{15}N abundance of the pool (Barraclough, 1991). Nonetheless, a correct estimate was obtained by Butterly et al. (2016).

An example of where the methodology based on ^{15}N enrichment (*E*) was flawed, involved two species of *Acacia* grown in a mixture of soil and perlite (1:1, v/v) in which the soil containing 0.3 g N kg^{-1} + perlite was initially amended with a dose of $Ca(^{15}NO_3)_2$ and then watered every 2 days with tap water (Nguyen et al., 2006). However, a non-fixing reference plant was not included in the experimental design, and so P_{atm} could not be estimated using Eq. 6. Instead *the amount* of fixed N_2 was calculated as *the difference* between the total N yield and the uptake of labelled N, thus ignoring the contribution of unlabelled N derived from the soil and tap water. Therefore the whole purpose of using ^{15}N enrichment to obtain a yield-independent estimate of P_{atm} was defeated by this yield-dependent approach which over-estimated P_{atm}.

These three examples illustrate that caution should be exercised when interpreting the results of (*E*) studies designed to estimate P_{atm} under e[CO_2].

2.4.3. ^{15}N natural abundance (NA)

For the *NA* method, P_{atm} is estimated by Eq. 8.

$$P_{\text{atm}} = \frac{\delta^{15}\text{N}_{\text{reference plant}} - \delta^{15}\text{N}_{\text{N2-fixing plant}}}{\delta^{15}\text{N}_{\text{reference plant}} - \text{B}} \quad (8)$$

where δ^{15}N and B were defined previously (Section 2.3.2.).

Several approaches have been used to estimate the B value. It is generally accepted that the B value should be determined experimentally under conditions where the legume or actinorhizal plant is wholly dependent on N_2 fixation. If the effect of e[CO_2] on legume symbiotic dependence is to be studied, then B should be determined at both a[CO_2] and e[CO_2]. Thomas et al. (1991) found no significant difference in the δ^{15}N values of 71-day-old Gliricidia grown in N-free sand culture between a[CO_2] and e[CO_2] treatments (see footnote, Table 1).

B is sometimes assumed to be zero (e.g. Guo et al., 2013), thus assuming the absence of isotopic fractionation during N_2 fixation and translocation in the plant. In the case where B = 0, Eq. 8 is equivalent to Eq. 6, since *E* and δ^{15}N are linearly related (Chalk et al., 2015). Published values of B for a particular N_2-fixing association have also been used, but it is preferable for this value to be determined experimentally by the same research group for the same cultivar (e.g. Lilley et al., 2001). However this is not always the case as Hoosbeek et al. (2011) and Millett et al. (2012) used published B values for black alder that were determined by another group 23 years previously, possibly using a different provenance. Another approach is to set the B value at the lowest δ^{15}N value recorded for that particular N_2 fixing association, which assumes that this value represents the value for a fully symbiotic plant (West et al., 2005). Yet another method is to use an average δ^{15}N value of soil (Watanabe et al., 2013). However the soil δ^{15}N signature reflects the net time-integrated value of the isotopic fractionations attendant on the myriad of biological N transformations occurring within the soil matrix, and therefore it is naive to assume that isotopic fractionation associated with a wholly symbiotic plant is reflected in the δ^{15}N signature of the soil. A full discussion of the limitations of the *NA* methodology including errors associated with the B value can be found in Unkovich et al. (2008).

In a long-term experiment involving annual measurement for 10 years (1998 to 2007 inclusive) of P_{atm} of a native leguminous vine, Elliott's milkpea, Hungate et al. (2014) used the δ^{15}N of the legume (–2.2 ‰) measured at the start of the experiment as the B value. The experiment began by applying a single dose of $(^{15}NH_4)_2SO_4$ (0.18 g N m^{-2} at 0.999 atom fraction ^{15}N; Hungate et al., 2004) but over the years the isotope became diluted so that at the last harvest the δ^{15}N signatures in the leaves of the oak reference plants fell to 84.3 ± 4.2 ‰. Therefore on the basis of this value it is apparent that the value of B was not critical to the estimation of P_{atm}, as would be the case if the reference plant had a δ^{15}N value close to that of the atmosphere.

3. Response of symbiotic dependence (P_{atm}) to e[CO_2]

3.1. Studies with legumes grown in artificial media

3.1.1. ^{15}N enrichment (E)

Several studies of the effect of e[CO_2] have been conducted on the symbiotic dependence of white clover (Zanetti et al., 1998; Almeida et al., 2000) grown in sand culture, and of the woody legumes *Acacia melanoxylon* (Schortemeyer et al., 1999),*Robinia pseudoacacia* (Feng et al., 2004) and *Gliricidia sepium* (Thomas et al., 2000) grown in hydroponics or sand culture (Table 1). P_{atm} of white clover or black wattle was not significantly affected when seedlings were exposed for 30-36 days to e[CO_2] compared witha[CO_2], but there were marked depressing effects of increasing [NO_3^-] or stimulating effects of phosphorus [P] on P_{atm} at both CO_2 concentrations (Table 1). The depressing effect of mineral N on legume symbiotic dependence is well documented (e.g. Hamilton et al., 1991) as is the effect of acute P deficiency (Chalk, 2000). In contrast, a marked stimulating effect on P_{atm} was observed for 1-year-old black locust exposed to prolonged e[CO_2] for 16 weeks at 4 mM N (Feng et al., 2004; Table 1), which may point to an effect of plant age or [CO_2] exposure time on P_{atm} in the case of woody legumes. Increasing concentrations of N also depressed P_{atm} of Gliricidia, but whereas there was no difference between a[CO_2] and e[CO_2]treatments at 1 mM N, P_{atm} at e[CO_2] was significantly higher than a[CO_2] at 10 mM N (Table 1).

3.1.2. ^{15}N natural abundance (NA)

Short-term exposure studies (56-71 d) have been conducted at *NA* on the effect of e[CO_2] on the symbiotic dependence of the woody legumes *Gliricidia sepium* (Thomas et al., 1991) and *Robinia pseudoacacia* (Olesniewicz and Thomas, 1999) grown in sand culture (Table 1). In both cases there was a significant increase in P_{atm} at e[CO_2] in the presence of 1-7 mM N, which was consistent with the result obtained for Gliricidia by Thomas et al. (2000) at 10 mM N. In the case of black locust both the absolute values of P_{atm} and the increase in the absolute values (18-22 %) were similar, whether the exposure time was short (56 d, *NA*) or long (112 d, *E*), respectively (Table 1). There was also a significant positive response of P_{atm} of black locust to inoculation with mycorrhiza both at a[CO_2] and e[CO_2], as previously reviewed by Chalk et al.(2006).

Table 1

^{15}N enrichment (*E*) and ^{15}N natural abundance (*NA*) studies to quantify the effect of e[CO_2] on P_{atm} of legumes grown in artificial media in growth chambers.

Legume[a]	Medium	[CO_2][b] exposure		[N][c]	[P]	$E_{fertilizer}$[e]	P_{atm} (%)[g]	Reference
		(µmol mol^{-1})	Duration(d)					
E studies								
White clover	Sand	35 or 60 Pa	36	0		1.00	100	Zanetti et al., 1998
				7.5			31	
Black wattle	Hydroponics	350 or 700	35	3		10	80-85	Schortemeyer et al., 1999
				100			13-15	
White clover	Sand	35 or 70 Pa	30	1.5 mM	0 mM	0.90	25-30	Almeida et al., 2000
					2 mM[d]		70-72	
Gliricidia		350, 700	100	0		+ 20 ‰	100	Thomas et al., 2000
				1 mM			88, 90	
				10 mM			19, 47	
Black locust		350	112	4 mM		8.93	20	Feng et al., 2004
		700					42	
NA studies					±Mycorrhiza	$\delta^{15}N$ (‰)		
Gliricidia	Sand	350 µbar	71	7 mM		–2.64	34[f]	Thomas et al., 1991
		650 µbar					56[f]	
Black locust		350	56	1 mM	–	–20	25a	Olesniewicz and Thomas, 1999
					+		57b	
		710			–		45c	
					+		63d	

[a] White clover, *Trifolium repens*; Black wattle, *Acacia melanoxylon*; Gliricidia, *Gliricidia sepium*; Black locust, *Robinia pseudoacacia*;
[b] 1 Pa = 10 µbar = 9.869 µmol mol^{-1}
[c] 7.5 mol N m^{-3} as NH_4NO_3; 3 and 100 mmol N m^{-3}as KNO_3; 1.5 mM urea; 1, 4, 7, 10 mM NH_4NO_3
[d] KH_2PO_4
[e] Excess atom % ^{15}N
[f] Leaf $\delta^{15}N$ values for 7 mM NH_4NO_3 at 350 and 650 µbar [CO_2] were –2.24±0.10 and –1.86±0.09 ‰, respectively, while the corresponding B values (plants 100% dependent on N_2) were –1.45±0.02 and –1.25±0.09‰. Values of P_{atm} calculated according to Eq. 3.
[g] Means followed by different letters are significantly different (P<0.05)

3.2. Soil-based studies

3.2.1. Endophytic and free-living ($^{15}N_2$)

As far as we are aware only one study has been reported where $^{15}N_2$ was used to directly measure the effect of e[CO_2] on endophytic BNF in a C_3 sedge (*Spartina patens*) and a C_4 grass

(*Scirpus olneyi*) growing in anaerobic sediment. Plants that had been exposed to ambient or e[CO_2] (360 and 660 µmol mol^{-1}, respectively) for 4 months in open top chambers were then exposed to $^{15}N_2$ for 72 h (Dakora and Drake, 2000). In both species, the incorporation of ^{15}N into plant tissue increased significantly at e[CO_2], indicating a positive response of the resident endophytic diazotrophs. In addition, incorporation of ^{15}N into plant-free sediment significantly increased at e[CO_2] indicating that free-living heterotrophs responded positively to the higher atmospheric CO_2 concentration. In contrast, Garten et al. (2008) found no effect of e[CO_2] compared with a[CO_2] on $^{15}N_2$ fixation in dry or wet glucose-amended surface soils incubated aerobically for 36 days or anaerobically for 15 days at 20^{o}C.

3.2.2. ^{15}N enrichment (E)

The majority of studies have been carried out with pasture or forage legumes with only single studies each for a woody legume and a grain legume (Table 2). The symbiotic dependence of the woody legume, sweet acacia, increased markedly to exposure to e[CO_2] for 13 months (Polley et al., 1997). In several studies, the interacting effects of soil mineral N and [CO_2] on $P_{atm}$was estimated for field pea, white clover and alfalfa (Butterly et al., 2016; Hartwig et al., 2002; Lüscher et al., 2000, respectively). At ambient concentrations of mineral N there was little or no response of P_{atm} to e[CO_2], but at increased [N] the response was positive, particularly for alfalfa and field pea (Table 2), which was consistent with results found for black locust and Gliricidia grown in artificial media (Table 1). For perennial pasture cut 3 to 4 times each year for 1 to 5 years the response of P_{atm} of the legumes in the sward to e[CO_2] was generally not significant (Table 2).

3.2.3. ^{15}N natural abundance (NA)

Several studies of the effect of e[CO_2] on N_2-fixing plants have provided only qualitative data based on a comparison of the $\delta^{15}N$ values of leaves (Table 3). Ariz et al. (2015) found that alfalfa leaf $\delta^{15}N$ was the most responsive plant parameter (cf. stems, roots or nodules) to e[CO_2], temperature and water availability. In the two cases where data were available (Tu et al., 2009; Vogel et al., 1997), leaf $\delta^{15}N$ values of N_2-fixing plants were significantly less than soil $\delta^{15}N$ values (Table 3) which could indicate an input from BNF. Except for black alder, leaf $\delta^{15}N$ values of a range of grain and forage legumes were less at elevated compared with ambient [CO_2] (Table 3) which may indicate additional BNF inputs due to e[CO_2].

A range of quantitative studies of [CO_2] on P_{atm} have been conducted with grain, forage, native and woody legumes (Table 4), which have revealed some inconsistencies in results obtained, including a few negative effects of e[CO_2]. A woody actinorhizal species, black alder, showed little response to e[CO_2] when grown alone or in mixtures with other species (Hoosbeek et al., 2011; Millett et al., 2012). Of four native legumes, two gave a positive response and two a negative response to e[CO_2] at low concentrations of mineral N, while when N was added to the plots the two species that showed a negative response at low N showed no response to e[CO_2] (West et al., 2005). On the other hand, one of the species (Amorpha) which showed a positive response at low N gave a negative response to e[CO_2] at high N (West et al., 2005). Although a native leguminous vine, Elliott's milkpea, showed year-to year variation in P_{atm}, there was little effect of e[CO_2] on annual estimates of symbiotic dependence in a long term (10 y) experiment (Hungate et al., 2014).

The forage legumes sub-clover (Lilley et al., 2001) and barrel medic (Guo et al., 2013; Lam et al., 2012 a) all showed marked positive responses to e[CO_2], while white clover had a marked negative response (Watanabe et al., 2013). Grain legumes similarly had a variable response to e[CO_2] (Table 4). Soybean had either a strong positive or no response to e[CO_2] depending on the cultivar (Lam et al., 2012 c). The symbiotic dependence of chickpea and field pea did not respond significantly to e[CO_2] across two (Lam et al., 2012 a) or three (Armstrong et al., 2015) soil types, regardless of soil P supply (Lam et al., 2012 a) or year (Armstrong et al., 2015) (Table 4).

4. N transfer in mixed swards in response to e[CO_2]

In a mixed stand of legume and grass the proportion of non-legume N derived from the transfer of biologically-fixed N can be estimated by ^{15}N dilution methods (reviewed by Chalk et al., 2014). The indirect (E) method requires the non-legume to be grown in a pure as well as the mixed stand. The proportion of non-legume N derived from the transfer of legume biologically-fixed N ($P_{\text{non-legume}(\hookleftarrow\text{legBNF})}$) is estimated by Eq. 9.

$$P_{\text{non-legume}(\hookleftarrow\text{legBNF})} = 1 - \frac{E_{\text{non−legume in mixed stand}}}{E_{\text{non−legume in pure stand}}} \qquad (9)$$

$P_{\text{non-legume}(\hookleftarrow\text{legBNF})}$ in a mixed stand of white clover and perennial ryegrass cut five times during each season (May-October) in 1993 and 1994 fluctuated during the season (range of 10-60 % per cut), but was consistently higher at each cut at 700 compared with 350 ppm [CO_2], indicating that the e[CO_2] treatment stimulated N transfer (Soussana and Hartwig, 1996). In contrast, Zanetti and Hartwig (1997) found identical values of 34 % for N transfer ($P_{\text{non-legume}(\Leftarrow\text{legBNF})}$) from white clover to ryegrass averaged across two seasons (1993, 1994) with 3 or 4 cuts per season, respectively, when exposed to pCO_2 of 35 or 60 Pa. Therefore the limited experimental data do not allow definitive conclusions to be reached.

Table 2

^{15}N enrichment (*E*) studies to quantify the effect of e[CO_2] on P_{atm} of soil-grown legumes.

Legume^{a}	Facility^{b}	[CO_2]^{c} exposure		Reference plant^{d}	Year	[N]	P_{atm} (%)		Reference
		(µmol mol^{-1})	Duration				a[CO_2]	e[CO_2]	
White clover	FACE	35, 60 Pa	Seasonal (3-4 cuts y^{-1})	Perennial ryegrass	1993	10^{f}	53	70	Zanetti et al., 1996
					1994	14^{f}	54	57	
					1995		54	61	
					1993	42^{f}	36	48	
					1994	56^{f}	27	35	
					1995		30	33	
Sweet acacia	GH	385, 690	13 mo	Little bluestem			36	69	Polley et al., 1997
Alpine clover	OTC	355, 680	Seasonal	Dandelion	92-95		58±5	64±7	Arnone, 1999
Alfalfa	FACE	35, 60 Pa	Seasonal (4 cuts)	Ineffective nodulating isolines	1995	2.5^{e}	82	88	Lüscher et al., 2000
						20^{e}	21	41	
White clover			Seasonal (4 cuts y^{-1})	Perennial ryegrass	1995-1998	14^{f}	80	81	Hartwig et al., 2002
						56^{f}	62	69	
Four legumes in perennial pasture	SACC	356, 600	Seasonal, March-November	Non-legumes in plot	1995-		93±1	91±4	Niklaus and Körner, 2004; Niklaus et al., 1998
					1998		88±3	99±2	
					1999		96±3	100±2	
Field pea	FACE	390, 550	15 weeks	Wheat		5^{g}	75	80	Butterly et al., 2016
						90^{g}	8	19	

[a] White clover, *Trifolium repens*; Sweet acacia, *Acacia smallii*; Alpine clover, *Trifolium alpines*; Alfalfa, *Medicago sativa*; Four legumes, Bird's-foot trefoil, *Lotus corniculatus*, Horseshoe Vetch, *Hippocrepis comosa*, White clover and Zigzag clover, *Trifolium medium*; Field pea, *Pisum sativum*

[b] FACE, Free air CO_2 enrichment; GH, greenhouse; SACC, Screen-aided CO_2 control; OTC, open top chamber

[c] 1 Pa = 9.869 µmol mol^{-1}

[d] Perennial ryegrass, *Lolium perenne*; Little bluestem, *Schizachyrium scoparium*; Non-legumes in plot were dominated by meadow brome (*Bromus erectus*); Dandelion, *Leontodon helveticus*; Wheat, *Triticum aestivum*

[e] g m^{-2}cut^{-1}

[f] g m^{-2} y^{-1}

[g] mg kg^{-1} soil

Table 3

δ^{15}N values of the leaves of N_2-fixing plants under a[CO_2] and e[CO_2].

N_2-fixing plants[a]	Facility[b]	[CO_2][c] exposure		δ^{15}N (‰)	Leaf δ^{15}N (‰)		Reference
		(μmol mol^{-1})	Duration	Soil	a[CO_2]	e[CO_2]	
Black alder	OTC	35, 70 Pa	160 d	+2.6	–1.5	–1.6	Vogel et al., 1997
Purple prairie clover	FACE	Ambient, 55 Pa	Not specified		+0.2	–0.5	BassiriRad et al., 2003
Leadplant					0	–0.6	
Roundhead bushclover					+0.8	–0.7	
Groundnut	OTC	376, 730	124 d	+4.4±0.2	+2.7	+2.3	Tu et al., 2009
Alfalfa	TGG	400, 700	30 d		–0.1	–0.6	Ariz et al., 2015

[a] Black alder, *Alnus glutinosa*; Purple prairie clover, *Petalostemum villosum*; Leadplant, *Amorpha canescens*; Roundhead bushclover; *Lespedeza capitata*; Groundnut, *Arachis hypogaea*; Alfalfa, *Medicago sativa*, was grown in an artificial mixture of vermiculite + perlite at ambient temperature under well watered conditions

[b] OTC, open top chamber; FACE, Free air CO_2 enrichment; TGG, temperature gradient greenhouse

[c] 1 Pa = 9.869 μmol mol^{-1}

Table 4

^{15}N natural abundance (*NA*) studies to quantify the effect of e[CO$_2$] on P_{atm} of soil-grown N$_2$-fixing plants.

N$_2$-fixing plants[a]	Facility[b]	[CO$_2$] exposure		Reference plant[d]	B-value		Treatment		P_{atm} (%) at [CO$_2$]		Reference
		(µmol mol^{-1})[c]	Duration		(‰)	Method[e]			a[CO$_2$]	e[CO$_2$]	
Subclover	Tunnel	380, 690	348 d	Phalaris	–0.9	2	Monoculture		59±6	71±4	Lilley et al., 2001
							Mixture		71±5	82±1	
Leadplant	FACE	368, 560	1998-Aug. 2002	Non-legumes in plot	–1.2	3	0, 4 g N m^{-2} y^{-1}		73, 63	95, 45	West et al., 2005
Roundhead bushclover					–2.1				62, 52	96, 62	
Wild lupine					–1.7				100, 73	78, 73	
Purple prairie clover					–1.0				97, 58	72, 55	
Red clover		390, 690	1 yr	Average 4 species	–1.6	1	Average 2 legumes 2004		73 ± 4	85 ± 6	Garten et al., 2008
Chinese bushclover					–2.7						
Black alder		Ambient, 580	3 yr	Birch	–2.6	2	Monoculture		61	60	Hoosbeek et al., 2011
		380, 580	4 yr	Birch, Beech	–1.9	2	Monoculture		60	59	Millett et al.,2012
							Mixture		62	68	
Barrel medic	GH	390, 700	0-pod-fill	Wheat	–0.4	2	± [P]	Vert-osol[g]	38 (56)[i]	61 (57)[i]	Lam et al., 2012 a
Chickpea					–1.6		± [P]		31 (28)[i]	15 (30)[i]	
Field pea					–0.6		± [P]		47 (49)[i]	25 (33)[i]	
Barrel medic					–0.4		± [P]	Calc-ar-osol[g]	21 (29)[i]	52 (45)[i]	
Chickpea					–1.6		± [P]		37 (31)[i]	38 (43)[i]	
Field pea					–0.6		± [P]		46 (48)[i]	53 (54)[i]	
Soybean, cvs. Zhonghuang 13 & 35	FACE	415, 550	111 d		–1.8	Not stated	Z. 13		59	79	Lam et al., 2012 c
							Z. 35		31	23	
Barrel medic	OTC	390, 750		Mutants	0	4			40±1	65±1	Guo et al., 2013
White clover	FACE	Ambient, 475	13 yr	Ryegrass	–1.8	5	Mixture		90±4	72±7	Watanabe et al., 2013
Elliott's milkpea	OTC	Am, Am + 350	10 yr	3 Oak sp.	–2.2	3			91-99[f]	88-100[f]	Hungate et al., 2014
Field pea	FACE	390, 550	0-Flow-ering	Wheat	–0.3	Not stated	Calcarosol[g]		50 (70)[h]	59 (71)[h]	Armstrong et al., 2015
							Vertosol[g]		55 (63)[h]	65 (67)[h]	
							Chromosol[g]		9 (26)[h]	11 (19)[h]	

[a] Subclover, *Trifolium subterraneum*; Leadplant, *Amorpha canescens*, Roundhead bushclover, *Lespedeza capitata*; Wild lupine, *Lupinus perennis*, Purple prairie clover, *Petalostemum villosum*; Red clover, *Trifolium pretense*; Chinese bushclover, *Lespedeza cuneata*; Black alder, *Alnus glutinosa*, Barrel medic, *Medicago truncatula*; Chickpea, *Cicer arietinum*; Field pea, *Pisum sativum*; Soybean, *Glycine max*;White clover, *Trifolium repens*; Elliott's milkpea, *Galactia elliottii*

[b] OTC, open top chamber; FACE, Free air CO$_2$ enrichment; GH, greenhouse

[c] Am., Ambient

[d] Phalaris, *Phalaris aquatica*; 4 species, *Plantago lanceolata, Andropogan virginicus, Festuca pratense, Dactylis glomerata*; Birch (silver), *Betula pendula*, Beech (European), *Fagus sylvatica*; Wheat, *Triticum aestivum*; Mutants, non-N$_2$-fixing *dnf1-1* and *dnf1-2* of *M. truncatula*; Ryegrass (perennial), *Lolium perenne*; Oak spp., *Quercusmyrtifolia, Q. geminata, Q. chapmanii*

[e] 1, N-free medium; 2, published data; 3, most negative value recorded; 4, zero; 5, national soil average

[f] Range of annual estimates 1998-2007 inclusive

[g] Soil type

[h] Data without parentheses are year 2010, Data in parentheses are year 2011

[i] Data in parentheses are for high P input

5. Response of N yield and symbiotic performance (N yield × P_{atm}) to e[CO_2]

The response of harvested N yield to e[CO_2] was positive in a majority of studies (Table 5). However, there were instances where N yield was not affected by e[CO_2] (Millett et al., 2012; Zanetti et al., 1996), and in the latter case this occurred at the highest level of mineral N addition. A negative response of N yield to e[CO_2] was found for black wattle at a low level of mineral N addition, but became strongly positive when N addition was high (Schortemeyer et al., 1999).

The response of the amount of fixed N to e[CO_2] was somewhat inconsistent, with large positive increases, no effect or a negative effect (Table 5). The positive effect was quite marked when both N yield and P_{atm} increased simultaneously in response to e[CO_2] (e.g. Feng et al., 2004; Polley et al., 1997; Thomas et al., 2000). On the other hand, the amount of fixed N remained unresponsive to e[CO_2] when both N yield and P_{atm} were themselves unresponsive (Millett et al., 2012). There were few instances where the amount of fixed N responded negatively to e[CO_2] and this occurred when N yield was depressed (e.g. Schortemeyer et al., 1997).

6. Factors interacting with the response of N yield, P_{atm} and fixed N to e[CO_2]

Several biotic, edaphic and agronomic variables have been included in studies of the effect of e[CO_2] on N yield, symbiotic dependence and symbiotic performance (Table 6). Some of these variables have a direct effect on N yield and P_{atm} and thus the amount of fixed N. For example, it is well known that mineral N suppresses P_{atm}while P deficiency can depress N yield and also P_{atm} if the deficiency is severe (Chalk, 2000). A consistent result previously observed in several studies is that e[CO_2] can moderate the depressing effect of mineral N on P_{atm}. In addition to mineral nutrition, biotic (e.g. mycorrhiza), and agronomic (cultivar, cropping system) can interact with the response of N_2-fixing plants to e[CO_2]. The effect of cropping system (monoculture vs. swards or intercrops) is exerted through the effect of the non-legume in scavenging available soil N and thus enhancing P_{atm} of the N_2-fixing component. Several examples of the ways in which the above variables interact with the effect of e[CO_2] on N yield, P_{atm} and fixed N have already been discussed (Tables 1, 2, 4, 5).

Table 5

Symbiotic performance of N_2-fixing plants under ambient and $e[CO_2]$.

N_2-fixing plants[a]	Treatment[b]	N yield (mg)		P_{atm} (%)		N fixed (mg)		Reference
		$a[CO_2]$[c]	$e[CO_2]$[c]	$a[CO_2]$[c]	$e[CO_2]$[c]	$a[CO_2]$[c]	$e[CO_2]$[c]	
Woody species								
Gliricidia	+ N	553	647	34	56	188	362	Thomas et al., 1991
	10 mM N	941	1559	19	47	179	733	Thomas et al., 2000
Sweet acacia		28[g]	43[g]	36	69	10[g]	30[g]	Polley et al., 1997
Black wattle	+3 N	34	25	86	81	30	20	Shortemeyer et al., 1999
	+100 N	270	377	14	12	35	45	
Black locust	–Mycorrhiza	40	66	25	46	10	30	Olesniewicz and Thomas, 1999
	+Mycorrhiza	122	207	57	64	70	132	
	+ N	106	161	20	42	21	68	Feng et al., 2004
Black alder	Monoculture	12[g]	14[g]	60	59	7[g]	8[g]	Millett et al., 2012
	Mixed culture	18[g]	22[g]	62	68	11[g]	15[g]	
Grain legumes								
Soybean	Cultivar Z. 13	284[d]	349[d]	59	79	166[d]	275[d]	Lam et al., 2012 c
	Cultivar Z. 35	249[d]	295[d]	31	23	75[d]	69[d]	
Chickpea	Vertosol –P	23	34	31	15	7	5	Lam et al., 2012 a
	Vertosol +P	34	54	28	30	10	16	
	Calcarosol –P	61	84	37	38	23	32	
	Calcarosol +P	75	87	31	43	23	37	
Field pea	Vertosol –P	33	45	47	25	16	11	
	Vertosol +P	60	78	49	33	29	26	
	Calcarosol –P	67	80	46	53	31	42	
	Calcarosol +P	85	110	48	54	41	59	
	+5 N	299	394	75	80	224	317	Butterly et al., 2016
	+ 90 N	342	421	8	19	28	82	
Forage legumes								
White clover	1993, +10 N	40.6[e]	49.8[e]	53	70	21.4[e]	35.1[e]	Zanetti et al., 1996
	1994, +14 N	50.4[e]	55.6[e]	54	57	27.4[e]	31.6[e]	
	1995, +14 N	50.6[e]	48.8[e]	54	61	27.4[e]	30.0[e]	
	+ 14 N	18.9[e]	28.9[e]	80	81	15.2[e]	23.4[e]	Hartwig et al., 2002
	+ 56 N	17.1[e]	28.9[e]	62	69	10.6[e]	19.8[e]	
Barrel medic	Vertosol –P	22.9	31.0	38	61	8.7	18.9	Lam et al., 2012 a
	Vertosol +P	51.0	87.4	56	57	28.6	49.8	
	Calcarosol –P	56.2	85.3	21	52	11.8	44.4	
	Calcarosol +P	70.5	103.5	29	45	20.4	46.6	
				40	65	9.1[f]	25.3[f]	Guo et al., 2013

[a] See Tables 1, 2 and 4 for botanical names

[b] N, nitrogen fertilizer; Z, Zhonghuang;P, phosphorus fertilizer

[c] See Tables 1, 2 and 4 for $a[CO_2]$ and $e[CO_2]$ [d] Data are kg N ha^{-1}

[e] Data are g N $m^{-2}y^{-1}$

[f] Data are mg N $plant^{-1}$

[g] Data are g N

Table 6

Factors interacting with the response of N_2-fixing plants to e[CO_2].

N_2-fixing plant[a]	Factor	Variable[b]	Reference
Black locust	Biotic	Mycorrhiza	Olesniewicz and Thomas, 1999
White clover	Edaphic	Phosphorus nutrition	Almeida et al., 2000
1 forage, 2 grain			Lam et al., 2012 a
Alfalfa		Nitrogen availability	Lüscher et al., 2000
White clover			Hartwig et al., 2002
4 native legumes			West et al., 2005
Field pea			Butterly et al., 2016
1 forage, 2 grain		Soil type	Lam et al., 2012 a
Field pea			Armstrong et al., 2015
White clover	Agronomic	Cropping system	Soussana and Hartwig, 1996
			Zanetti and Hartwig, 1997
Sub clover			Lilley et al., 2001
Black alder			Millett et al., 2012
Soybean		Cultivar	Lam et al., 2012 c
Sub clover	Environmental	Temperature	Lilley et al., 2001
2 forage			Garten et al., 2008
Alfalfa			Ariz et al., 2015
2 forage		Water	Garten et al., 2008
Alfalfa			Ariz et al, 2015
Groundnut		Ozone	Tu et al., 2009

[a] See Tables 1, 2 and 4 for botanical names; 1 forage, Barrel medic, 2 grain, Chickpea, Field pea; 4 native legumes, Leadplant, Roundhead bushclover, Wild lupine, Purple prairie clover; 2 forage, Red clover, Chinese bushclover

[b] Cropping system, monoculture vs. mixed cropping

Environmental factors can also play a rolein the response of N_2-fixing plants to e[CO_2]. For example, Lilley et al. (2001) reported that P_{atm} of sub-clover increased by 12 % under e[CO_2], but decreased by 6 % when the temperature was increased by 3.4 $^{\mathrm{o}}$C above ambient. Based on qualitative data of differences in δ^{15}N values of the biomass of groundnut at maturity, Tu et al. (2009) concluded that increased concentrations of ozone [O_3] inhibited apparent N_2 fixation, but the response was moderated by e[CO_2]. Garten et al. (2008) studied the complex 3-way interaction of e[CO_2], temperature and water availability in two forage legumes over 3 years.In the first year there was a significant interaction between [CO_2] and watering regime, with P_{atm} greater under e[CO_2] in the wet treatment, but less under a[CO_2] in the dry treatment. However, the observations were inconsistent between years, with no main effects or interactions in the third year.

7. Conclusions

Various ^{15}N-based methods including the direct $^{15}N_2$ technique and indirect isotope dilution methods at enriched and natural abundance levels of ^{15}N have been applied to obtain quantitative estimates of the symbiotic dependence of a range of woody and herbaceous N_2-fixing plants. However, a fundamental understanding of the principles and assumptions on which each method is based is essential for the correct interpretation of results. With the ^{15}N natural abundance method, the B value represents a potential source of error, with several different approaches taken in its estimation, including the unsatisfactory assumptions that B = 0 or B = δ^{15}N of the soil. Therefore whenever possible, it is recommended that the B value be determined directly in an N-free medium. At the present time it appears that the results of the effect of e[CO_2] on P_{atm} using the *NA* technique may have been confounded by methodological errors.

In order to avoid the assumptions on which soil-cultured *E* and *NA* methods are based, the use of artificial growth media or exposure to $^{15}N_2$ are alternative techniques which could be strategically employed. Another approach would be to develop methodologies not based on the use of a reference plant. Indeed, two such approaches have been proposed including modeling the temporal change in soil ^{15}N enrichment (Chalk et al., 1996) and measuring the difference in the δ^{15}N signatures between nodulated legume roots and shoots (Wanek and Arndt, 2002).

The response of a N_2-fixing plant to an increase in a variable such as e[CO_2] will only be expressed to its full extent if other factors affecting plant N yield and symbiotic dependence are non-limiting. This has been illustrated in the present review with respect to plant mineral nutrition (N and P), biotic (e.g. mycorrhiza), agronomic (cultivar, cropping system) and environmental (e.g. temperature) factors. When one considers the large variation in the conditions under which experiments have been conducted such as period of exposure to [CO_2], type of facility, ^{15}N methodology applied, N_2-fixing system studied, stage of plant growth, growth medium, etc., it is understandable that difficulties may arise in interpretation of results.

In the meta-analysis of the effect of e[CO_2] on N dynamics in grain and pasture legumes (Lam et al., 2012 b), it was concluded that there was a 38 % increase in the amount of N fixed and a 10 % increase in symbiotic dependence. However, this analysis was based on only 9 studies that did not include woody species. The present review is based on the quantitative analysis of 26 studies supported by 4 qualitative studies across a range of woody, grain and pasture/forage N_2-fixing plants. This represents a significant additional coverage of the literature, and the variable and sometimes inconsistent results obtained from the analysis show that average values, while nevertheless useful, are not representative of the suite of responses possible in individual species.

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