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Cereal grain, rachis and pulse seed amino acid $\delta^{15}N$ values as indicators of plant nitrogen metabolism

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ABSTRACT

Natural abundance $\delta^{15}N$ values of plant tissue amino acids (AAs) reflect the cycling of N into and within plants, providing an opportunity to better understand environmental and anthropogenic effects on plant metabolism. In this study, the AA $\delta^{15}N$ values of barley (*Hordeum vulgare*) and bread wheat (*Triticum aestivum*) grains and rachis and broad bean (*Vicia faba*) and pea (*Pisum sativum*) seeds, grown at the experimental farm stations of Rothamsted, UK and Bad Lauchstädt, Germany, were determined by GC–C–IRMS. It was found that the $\delta^{15}N$ values of cereal grain and rachis AAs could be largely attributed to metabolic pathways involved in their biosynthesis and catabolism. The relative ^{15}N-enrichment of phenylalanine can be attributed to its involvement in the phenylpropanoid pathway and glutamate has a $\delta^{15}N$ value which is an average of the other AAs due to its central role in AA–N cycling. The relative AA $\delta^{15}N$ values of broad bean and pea seeds were very different from one another, providing evidence for differences in the metabolic routing of AAs to the developing seeds in these leguminous plants. This study has shown that AA $\delta^{15}N$ values relate to known AA biosynthetic pathways in plants and thus have the potential to aid understanding of how various external factors, such as source of assimilated N, influence metabolic cycling of N within plants.

1. Introduction

Natural abundance $\delta^{15}N$ values of bulk plant material reflect the net effect of a range of biochemical and environmental processes, which are difficult to disentangle from one another. These include the source of assimilated N, mycorrhizal associations, temporal and spatial variation in N availability, and changes in plant N demand (Robinson, 2001; Evans, 2001). Plants can be defined as N_2-fixing (leguminous) and non N_2-fixing. N_2-fixing plants can obtain their N from the atmosphere, whereas non N_2-fixing plants take up N from the soil. Since the $\delta^{15}N$ value of air is defined as 0‰, N_2-fixing plants tend to have $\delta^{15}N$ values close to 0‰ (van Klinken et al., 2000). Non N_2-fixing plants assimilate N from the soil in a variety of forms, primarily as inorganic NH_4^+ or NO_3^-. The $\delta^{15}N$ values of non N_2-fixing plants are generally assumed to be between 3‰ and 5‰, but studies of higher plants have shown that they can vary widely in their $\delta^{15}N$ values, from −8‰ to +18‰ (Kelly, 2000). Such variation can be due to a number of factors, namely: (i) the inherent variation in soil $\delta^{15}N$ values (Shearer et al., 1978), (ii) soil N availability (Mariotti et al., 1982; Evans, 2001), (iii) environmental factors, such as aridity (Heaton, 1987), (iv) anthropogenic addition of N to the soil through use of manure or artificial fertilisers (Choi et al., 2002), and (v) transpiration efficiency of net N uptake (Cernusak et al., 2009).

The N absorbed by plants must be converted into a form that can be used in protein biosynthesis. The NH_3 produced in the N_2 fixation reaction in legumes is retained by N-fixing cells in the symbiotic bacteria and reacts with glutamate to form glutamine, which can be absorbed by the plant (Schulten and Schnitzer, 1998). Leguminous plants can also absorb the NH_3 directly, via their root hairs, in the form of NH_4^+ ions and use the N to biosynthesise amino acids (AAs) and proteins. In non N_2-fixing plants, N is generally assimilated as NO_3^- or NH_4^+, but in ecosystems which are strongly N limited, it has been found that plants can also assimilate organic forms of N; primarily AAs (e.g. Näsholm et al., 1998; Weigelt et al., 2005). It seems, however, that the uptake of organic-N is of limited importance in agricultural situations, because plants compete poorly with microbes for AAs (Bardgett et al., 2003). NO_3^- taken up from the soil is reduced to NH_3 and this is then assimilated into glutamine by glutamine synthetase. NH_4^+ is incorporated directly into glutamine, using glutamate as a substrate. The amide-N of glutamine is transferred to α-ketogluturate by glutamate synthase and this forms glutamate (Forde and Woodall, 1995). The amide-N in glutamine is therefore the primary

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Table 1
Crop AA composition of (i) barley grains and rachis from the experimental farming stations of Rothamsted (ROT) and Bad Lauchstädt (BAD), (ii) bread wheat grains and rachis from Rothamsted and Bad Lauchstädt, and (iii) broad beans and peas from Bad Lauchstädt.

Crop species	Site	Year	Crop part	Manure treatment	Crop amino acid concentration ($mg\,g^{-1}$)								
					Phe	Glx	Pro	Ala	Asx	Val	Leu	Gly	Ser	Total
Barley	ROT	1962	Grain	Manure 35t/ha	4.6	24.9	11.2	3.5	6.1	3.2	6.1	3.5	3.5	66.6
				NIL	3.6	19.0	9.2	3.9	5.4	2.6	5.5	3.8	3.8	56.7
			Rachis	Manure 35t/ha	1.2	4.3	2.3	2.1	3.3	1.0	1.8	1.8	1.4	19.7
				NIL	0.9	2.6	1.4	1.5	2.2	1.2	1.4	1.4	1.1	14.0
	BAD	2008	Grain	Manure 30t/ha	1.8	9.7	3.8	1.9	4.0	1.9	3.0	2.0	1.5	29.6
				NIL	1.4	7.1	2.9	2.0	3.4	1.6	2.5	2.0	1.6	24.5
			Rachis	Manure 30t/ha	0.8	3.4	1.6	1.0	1.5	0.7	1.2	0.9	0.9	12.2
				NIL	0.3	1.1	0.7	0.7	1.1	0.5	0.6	0.6	0.6	6.6
Bread wheat	ROT	1965	Grain	Manure 35t/ha	4.9	36.8	12.3	3.8	5.5	3.4	6.8	4.3	3.0	80.7
				NIL	4.6	32.4	10.7	3.2	5.5	3.1	6.3	3.7	3.9	73.4
			Rachis	Manure 35t/ha	1.0	2.6	1.5	1.6	2.3	1.2	1.6	1.5	1.3	14.9
				NIL	0.8	0.9	1.2	0.8	0.7	0.8	1.4	0.7	0.4	7.7
	BAD	2008	Grain	Manure 30t/ha	2.2	15.3	4.6	1.5	3.1	1.1	3.1	1.8	1.4	34.2
				NIL	1.9	13.9	4.5	1.6	3.0	1.2	2.8	1.9	1.7	32.4
			Rachis	Manure 30t/ha	0.4	1.4	0.7	0.8	1.1	0.4	0.7	0.7	0.6	7.0
				NIL	0.3	1.1	0.7	0.7	0.8	0.2	0.6	0.7	0.5	5.9
Broad beans	BAD	2008	Pulse	Manure 30t/ha	5.8	26.0	6.5	6.0	17.8	5.0	10.3	6.0	7.6	91.0
				NIL	6.1	27.2	6.8	5.8	23.0	4.8	10.8	6.0	7.7	98.3
Peas	BAD	2008	Pulse	Manure 30t/ha	6.2	26.1	5.6	4.8	17.0	4.5	9.0	4.6	5.3	83.1
				NIL	5.4	23.3	5.4	5.3	14.6	3.4	8.6	5.0	6.0	77.0

source of N for the amino groups of all of the other AAs found in plants, via transamination reactions between glutamate and corresponding α-keto acids. Any deviation observed in the $\delta^{15}N$ values of other AAs from the $\delta^{15}N$ value of glutamate can therefore be attributed to isotopic fractionation occurring during subsequent primary and secondary N metabolism within the plant. Such reactions include reductions, amidations, transaminations, hydrolyses and lyase reactions; all of which have the potential for N kinetic and/or thermodynamic isotope effects.

Individual AA $\delta^{15}N$ values in plant protein will reflect the cycling of N into and within plants and could thus aid understanding of environmental and anthropogenic effects on plant metabolism. Natural abundance AA $\delta^{15}N$ values have been determined previously in only a handful of plants. These studies include the determination of AA $\delta^{15}N$ values in: (i) roots and shoots of grassland perennials to examine the effects of land use and fertiliser practices (Bol et al., 2002; Ostle et al., 1999); (ii) whole wheat protein hydrolysates to investigate the ecotoxicological effects of ozone at a molecular level (Hofmann et al., 1995); (iii) spinach, komatsuna and soybean leaves and roots (Yoneyama and Tanaka, 1999); (iv) mangrove leaves to understand more about ecosystem dynamics and nutrient availability in past stands (Smallwood et al., 2003); and (v) rapeseed leaves to understand leaf metabolic fluxes (Gauthier et al., 2013). The lack of replicates in most of these studies and the low number of plant species sampled means that they are of limited use for interpretation of plant N cycling. Nonetheless, although these studies do not provide a comprehensive set of plant AA $\delta^{15}N$ values, they do indicate the extent of variation in plant AA $\delta^{15}N$ values, which have the potential to be linked with metabolic pathways within the plant. The study by Gauthier et al. (2013) compared modelled $\delta^{15}N$ values of free AAs in leaves with measured values, to explain isotopic fractionation due to the dynamics of N metabolism. Gauthier et al. (2013) followed the metabolic model developed by Tcherkez (2011), which uses steady-state equations to predict AA $\delta^{15}N$ values. The AA $\delta^{15}N$ values determined in previous studies and those modelled by Tcherkez (2011) are discussed below in more detail, in relation to the results from this study.

In this study, the proteinaceous AA $\delta^{15}N$ values of barley (*Hordeum vulgare*) and bread wheat (*Triticum aestivum*) grains and rachis and broad bean (*Vicia faba*) and pea (*Pisum sativum*) seeds, grown at the experimental farm stations of Rothamsted, UK and Bad Lauchstädt, Germany, were determined by gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS). Each sample represents the combined cereal grains, rachis or pulse seeds from 10 plants randomly sampled along a central transect of each farming plot (for cereals ca. 200–300 grains and for pulses ca. 30–50 seeds). Table 1 gives details of the plant tissues. Details of the climate and soil types of the experimental plots are given in Fraser et al. (2011, Table 1). Determination of cereal grain, rachis and pulse AA $\delta^{15}N$ values will aid our understanding of plant N cycling in: (i) different crop species; (ii) in different parts of the same plant, and (iii) will also allow comparison of N cycling within cereals and legumes. The results obtained are interpreted in terms of a known metabolic framework of plant protein biosynthesis.

2. Results and discussion

The $\delta^{15}N$ values of nine AAs: alanine (Ala), aspartate (Asx), glutamate (Glx), glycine (Gly), leucine (Leu), phenylalanine (Phe), proline (Pro), serine (Ser) and valine (Val), were determined by GC–C–IRMS. These AAs account for 65% of the total AA–N in hulled barley grains (*H. vulgare*), 67% of the total AA–N in whole bread wheat grain (*Triticum spp.*), 53% of the total AA–N in broad beans (*V. faba*) and 51% of the total AA–N in peas (*P. sativum*; calculated from data from the FAO; see Table 1 for AA concentrations). During

hydrolysis, asparagine (Asn) and glutamine (Gln) are deamidated to form aspartate (Asp) and glutamate (Glu), respectively (Hill, 1965). This deamidation proceeds quantitatively and therefore the $\delta^{15}N$ value of Asx represents the amino-N of both Asp and Asn and the $\delta^{15}N$ value of Glx represent the amino-N of Glu and Gln.

In the following graphs, AA $\delta^{15}N$ values are normalised to Glx, to negate any differences in AA $\delta^{15}N$ values due to a difference in bulk plant tissue $\delta^{15}N$ value. The AAs are grouped according to metabolic relationships (explained in more detail below), in order of decreasing $\delta^{15}N$ value. Phe has a distinct metabolic pathway from the other AAs; Glx and Pro are closely related since the amino group of Pro comes from Glu; the amino group of Ala can come from Glu and also from γ-aminobutyric acid; the N from Asx is exchanged with many AAs, including Glu and Ala; Val and Leu are both branched-chain AAs; and Gly and Ser are both involved in photorespiration and can be biosynthesised from one another. The determined AA $\delta^{15}N$ values are summarised in Table 2.

2.1. Cereal grain amino acid $\delta^{15}N$ values

Fig. 1 shows the $\delta^{15}N$ values of barley and bread wheat grain AAs, normalised to Glx, from experimental farming plots at Rothamsted and Bad Lauchstädt. The $\delta^{15}N$ values of Phe (+8.0 ± 1.2‰), Pro (+2.0 ± 0.6‰) and Asx (+1.3 ± 0.4‰) are higher than the $\delta^{15}N_{Glx}$ value, whereas the $\delta^{15}N$ values of Leu (−2.2 ± 0.8‰), Gly (−1.5 ± 1.1‰) and Ser (−3.1 ± 1.2‰) are lower. Ala and Val have $\delta^{15}N$ values similar to that of $\delta^{15}N_{Glx}$ (within ± 1.3‰).

In cereals, most of the N stored in the grain comes from the senescing leaves, either derived from: (i) the proteolysis of leaf proteins, such as Rubisco (which contributes up to 50% of the total leaf protein and 30% of total leaf N), or (ii) the incorporation of foliar NH_4^+ into the amide group of Gln, using Glu as a substrate (John, 1992). N from AAs is transaminated onto Glu, which then provides the amide-N of Asn, since Asn is synthesised from Gln by asparagine synthetase. Thus, N arriving in the seeds in the phloem is predominantly in the form of Gln and Asn, however, the contribution of other imported AA to the total grain AA pool is difficult to quantify (Miflin and Lea, 1977). In the developing seed, the amide-N of Gln and Asn is released for incorporation into other AAs, generally through a transamination reaction via Glu (Pate, 1980). It can be assumed that the $\delta^{15}N$ value of the N source (in the case of *in situ* biosynthesis, the amide-N of Gln or Asn) and any isotopic fractionation associated with the biosynthetic pathway will determine the $\delta^{15}N$ value of the newly biosynthesised AAs in cereal grains.

Fig. 2 relates determined grain AA $\delta^{15}N$ values to known pathways of AA metabolism in cereal grains. Greater understanding of enzyme N fractionation and catabolic pathways is needed in order to fully explain the relative AA $\delta^{15}N$ values expressed in developing cereal grains, but the main differences can be rationalised based on known biosynthetic and metabolic pathways.

The significant role of Phe in the phenylpropanoid pathway, involved in the synthesis of a wide range of phenolic compounds (including lignin), can explain the relative ^{15}N-enrichment of Phe compared to the other AAs in cereal grains. The enzyme PAL, which catalyses the deamination of Phe in the first step of this phenylpropanoid pathway, is a branch-point enzyme and therefore the kinetic isotope effect associated with this deamination (Hermes et al., 1985) is likely to be expressed, leaving the residual Phe relatively enriched in ^{15}N. Such a significant ^{15}N-enrichment (average +8.0‰), compared to the $\delta^{15}N$ value of Glx in cereal grain protein, indicates the importance of the phenylpropanoid pathway in the biosynthesis of plant tissue (c.f. Cantón et al., 2005). Similarly high $\delta^{15}N$ values of Phe compared to other AAs have been observed by Hofmann et al. (1995) in whole wheat protein

Table 2
Crop AA $\delta^{15}N$ values for: (i) barley grains and rachis from the experimental farming stations of Rothamsted and Bad Lauchstädt, (ii) bread wheat grains and rachis from Rothamsted and Bad Lauchstädt, and (iii) broad beans and peas from Bad Lauchstädt. Numbers in brackets are the standard deviations associated with triplicate isotope determinations.

Crop species	Site	Year	Crop part	Manure treatment	Crop amino acid $\delta^{15}N$ values (‰)									
					Phe	Glx	Pro	Ala	Asx	Val	Leu	Gly	Ser	Bulk
Barley	ROT	1962	Grain	Manure 35t/ha	16.7 (0.3)	10.1 (0.1)	11.4 (0.2)	9.6 (0.2)	10.9 (0.2)	9.4 (0.8)	6.8 (0.4)	8.4 (0.3)	6.2 (0.2)	9.19
				NIL	10.7 (0.5)	3.6 (0.2)	4.9 (0.1)	3.2 (0.6)	4.4 (0.3)	3.9 (0.5)	0.7 (0.4)	0.7 (0.6)	−1.1 (0.2)	1.64
			Rachis	Manure 35t/ha	14.3 (0.7)	5.6 (0.0)	7.6 (0.1)	6.0 (0.4)	6.8 (0.1)	6.0 (0.4)	3.7 (0.2)	5.1 (0.3)	2.7 (0.1)	4.32
				NIL	10.6 (0.4)	1.3 (0.1)	3.4 (0.1)	1.6 (0.2)	2.8 (0.1)	2.6 (0.3)	−0.7 (0.2)	0.3 (0.1)	−1.5 (0.1)	−0.26
	BAD	2008	Grain	Manure 30t/ha	14.6 (0.2)	7.5 (0.1)	9.1 (0.3)	8.2 (0.2)	9.0 (0.2)	7.9 (0.3)	5.3 (0.6)	5.5 (0.4)	3.7 (0.3)	7.32
				NIL	10.6 (0.4)	1.3 (0.1)	3.4 (0.1)	1.6 (0.2)	2.8 (0.1)	2.6 (0.3)	−0.7 (0.2)	0.3 (0.1)	−1.5 (0.1)	−0.26
			Rachis	Manure 30t/ha	14.6 (0.2)	5.8 (0.1)	7.9 (0.4)	4.3 (0.3)	6.9 (0.2)	6.0 (0.3)	2.7 (0.4)	2.3 (0.4)	0.3 (0.7)	3.08
				NIL	11.8 (0.8)	−0.3 (0.3)	3.8 (0.2)	−2.1 (0.4)	0.6 (0.1)	3.7 (0.0)	−3.1 (0.3)	−6.6 (0.4)	−6.0 (0.4)	−1.80
Bread wheat	ROT	1965	Grain	Manure 35t/ha	14.6 (0.2)	7.5 (0.1)	9.1 (0.3)	8.2 (0.2)	9.0 (0.2)	7.9 (0.3)	5.3 (0.6)	5.5 (0.4)	3.7 (0.3)	7.36
				NIL	7.3 (0.4)	0.5 (0.2)	2.2 (0.2)	0.8 (0.4)	2.4 (0.4)	2.2 (0.3)	−2.1 (0.2)	−2.7 (0.2)	−3.0 (0.2)	0.74
			Rachis	Manure 35t/ha	15.7 (1.5)	9.1 (0.9)	10.6 (0.5)	7.4 (0.2)	9.2 (0.2)	8.8 (0.1)	5.9 (0.4)	3.4 (0.2)	2.1 (0.2)	5.30
				NIL	10.4 (0.9)	2.4 (1.4)	4.9 (0.4)	3.1 (0.2)	3.5 (0.9)	4.3 (0.3)	0.1 (0.7)	−1.0 (0.2)	−3.6 (1.2)	−2.50
	BAD	2008	Grain	Manure 30t/ha	12.8 (0.8)	4.0 (0.1)	6.7 (0.3)	5.8 (0.2)	5.4 (0.4)	6.1 (0.2)	3.0 (0.4)	3.6 (0.7)	2.9 (0.3)	4.91
				NIL	10.3 (0.4)	1.0 (0.2)	3.9 (0.2)	2.7 (0.3)	2.5 (0.1)	3.0 (0.2)	−0.3 (0.1)	0.7 (0.2)	−0.7 (0.5)	1.87
			Rachis	Manure 30t/ha	13.7 (0.2)	7.0 (0.2)	9.0 (0.2)	8.2 (0.2)	5.6 (0.2)	7.4 (0.4)	7.2 (0.1)	5.0 (0.6)	2.8 (0.2)	3.01
				NIL	11.9 (0.6)	3.3 (0.4)	6.1 (0.2)	5.2 (0.6)	1.7 (0.2)	3.6 (0.1)	5.0 (0.2)	1.2 (0.4)	−1.0 (0.3)	0.01
Broad beans	BAD	2008	Pulse	Manure 30t/ha	2.1 (0.3)	0.9 (0.2)	4.3 (0.1)	−0.9 (0.3)	2.4 (0.1)	0.3 (0.2)	−2.4 (0.2)	2.2 (0.2)	−2.5 (0.0)	−0.02
				NIL	1.3 (0.8)	1.2 (0.5)	3.1 (0.4)	0.1 (0.5)	1.9 (0.5)	−0.1 (0.4)	−4.4 (0.1)	1.7 (0.1)	−2.8 (0.5)	−0.7
Peas	BAD	2008	Pulse	Manure 30t/ha	−0.5 (0.7)	0.7 (0.3)	3.6 (0.3)	−0.4 (0.3)	1.0 (0.1)	0.4 (0.7)	−4.8 (0.6)	6.5 (0.6)	0.5 (0.4)	0.84
				NIL	0.3 (0.4)	0.9 (0.3)	3.8 (0.1)	0.2 (0.2)	2.3 (0.2)	0.6 (0.5)	−2.8 (0.1)	7.3 (0.2)	0.8 (0.4)	0.03

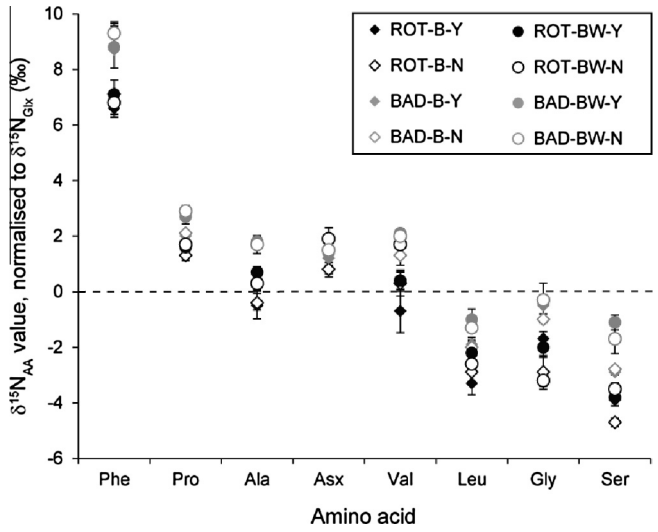

Fig. 1. Amino acid $\delta^{15}N$ values normalised to Glx from cereal grains grown on experimental plots at Rothamsted, UK, and Bad Lauchstädt, Germany. Error bars represent the standard deviation associated with triplicate isotope determinations. ROT = Rothamsted; BAD = Bad Lauchstädt; B = barley; BW = bread wheat; Y = manured; N = non-manured.

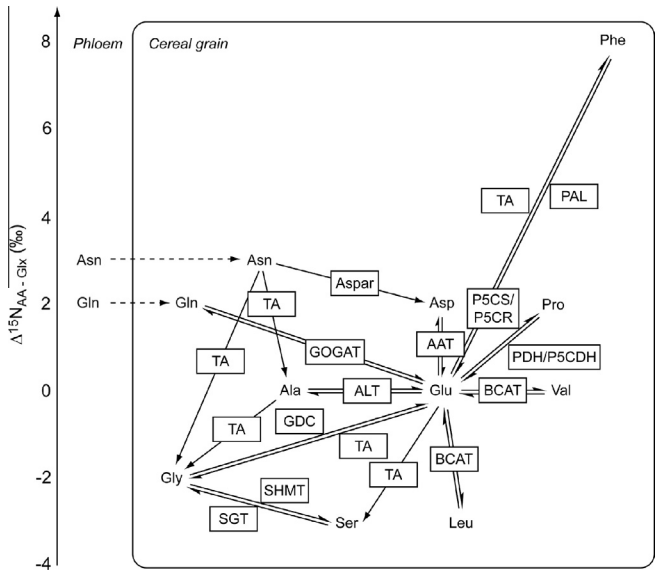

Fig. 2. The relationship between determined cereal grain AA $\delta^{15}N$ values and known pathways of AA metabolism in developing cereal grains. Enzymes are shown in boxes: AAT, aspartate transaminase; ALT, alanine transaminase; Aspar, asparaginase; BCAT, branched-chain aminotransferase; GDC, glycine decarboxylase; GOGAT, glutamate synthase; PAL, phenylalanine:ammonia lyase; P5CS, Δ^1-pyrroline-5-carboxylate synthetase; P5CR, Δ^1-pyrroline-5-carboxylate reductase; PDH, proline dehydrogenase; P5CDH, Δ^1-pyrrolidine-5-carboxylate dehydrogenase; SGT, serine:glyoxylate aminotransferase; SHMT, serine hydroxymethyltransferase; TA, transaminases.

hydrolysates; by Bol et al. (2002) in two grassland plants, *Lolium perenne* and *Juncus effusus*; and by Smallwood et al. (2003) in tall and dwarf *Red mangrove* trees. Interestingly, in the study carried out by Bol et al. (2002), *Brachythecium rutabulum*, a non-vascular plant, did not exhibit an enriched Phe $\delta^{15}N$ value. This supports the hypothesis that the relatively enriched $\delta^{15}N$ value of Phe is due to the metabolic pathway associated with lignin biosynthesis, since the vegetative parts of non-vascular plants do not contain lignin and therefore would not require such a high turnover of Phe.

The $\delta^{15}N$ value of Glx is a product of the net flux of N entering and leaving the Gln and Glu pools. The fact that it is more or less an average of the other AA $\delta^{15}N$ values indicates the centrality of Gln and Glu in AA metabolism. For example, while Glu provides the amino group for biosynthesis of Phe, Ala, Asp, Val, Leu and Gly, it also receives amino groups of AAs during catabolism.

Pro is biosynthesised from the cyclisation of Glu, without any breaking of bonds involving N. It is catabolised and its amino group transferred to Glu by the enzymes PDH and P5CDH. Since its $\delta^{15}N$ value is greater than that of Glu, it seems either that the kinetic isotope effect associated with the enzymes involved in the catabolism of Pro is greater than that associated with the biosynthesising enzymes, P5CS and P5CR, or there is a thermodynamic isotope effect associated with the biosynthesis of Pro, since the formation of a C–N bond tends to favour ^{15}N.

The measured $\delta^{15}N$ value of Ala is very similar to that of Glx (within ±1.8‰) in all of the cereal grains. This would suggest that there is little kinetic isotope effect associated with the transamination reaction of alanine aminotransaminase (ALT), which biosynthesises Ala from pyruvate and Glu, or that the reversible nature of this transamination reaction means that the $\delta^{15}N$ values of Glu and Ala reach equilibrium.

The $\delta^{15}N$ value of Asx in cereal grains is always slightly ^{15}N-enriched compared to that of Glx. This has been observed in other studies (Hare et al., 1991; Hofmann et al., 1995; Macko et al., 1987; Werner and Schmidt, 2002; Yoneyama et al., 1998). Tcherkez (2011) suggests that a relative ^{15}N-enrichment in Asp could be due to an inverse kinetic isotope effect associated with the transfer of the amino group from Glu onto oxaloacetate to form Asp. This is plausible since this reaction involves the reversible formation of a protonated Schiff base, which is more stable with Asp than Glu as a substrate. The relative ^{15}N-enrichment in Asx could also be due to the role of Asn as a transport metabolite to the developing grains, where its amino group is then incorporated into AAs within the grains, by transamination with α-keto acids. These transamination reactions are presumably accompanied by kinetic isotope fractionations, which discriminate against ^{15}N (Sieciechowicz et al., 1988).

It is unexpected that the $\delta^{15}N$ values of Val and Leu are very different from one another in cereal grains, since both receive their amino-N from the transamination of Glu (Binder et al., 2007). The $\delta^{15}N$ value of Leu is significantly ^{15}N-depleted relative to Val and this has been observed in studies of microorganisms (Macko et al., 1987) and plants (Bol et al., 2002; Hare et al., 1991; Hofmann et al., 1995). A study of branched-chain AA biosynthesis in spinach chloroplasts found that two forms of branched-chain aminotransferase (BCAT) exist; one described as valine aminotransferase with a clear preference toward the α-keto acid of Val and one termed leucine/isoleucine aminotransferase because it showed highest activity with the α-keto acids of Leu and Ile (Hagelstein et al., 1997). It is possible that the two forms of BCAT enzyme have different kinetic isotope effects associated with transamination, such that the transfer of ^{15}N is strongly discriminated against by leucine/isoleucine aminotransferase, leading to the relative ^{15}N-depletion in Leu. It is unusual, however, that the $\delta^{15}N$ value of Val is generally higher than that of Glx. This would suggest that there is another process, such as catabolism, that discriminates against ^{15}N and leaves Val relatively ^{15}N-enriched compared to its amino group donor, Glu.

Gly and Ser are integral to the photorespiratory cycle, which takes place in the leaves. In this process, the amino group of Gly comes from Glu, Asp or Ala from transamination with glyoxylate. The amino group of Ser then comes from the conversion of two molecules of Gly, generating an additional molecule of NH_3, which can enter the GS–GOGAT pathway to be reincorporated into Gln and then Glu (Keys et al., 1978). In non-photorespiratory tissues

(i.e. *in situ* synthesis in the grains), Gly is formed from Ser by the action of the enzyme SHMT. Ser is formed via the phosphorylation pathway, via transamination of 3-phosphohydroxypyruvate with Glu to form 3-phosphoserine, which is then converted into Ser (Ireland and Hiltz, 1995). The ^{15}N-depletion in Gly and Ser relative to the δ^{15}N value of Glx could be due either to: (i) ^{15}N-depletion in Gly formed during photorespiration and then imported into the grains, or (ii) a kinetic isotope effect associated with the *in situ* synthesis of Ser in the grains, involving transamination of Glu. The δ^{15}N value of Ser is always lower than that of Gly, which could be due to a thermodynamic isotope effect associated with the reversible reaction converting Ser to Gly. All other studies which have determined the AA δ^{15}N values of primary producers (algae and terrestrial plants) found that the δ^{15}N values of Gly and Ser were also ^{15}N-depleted relative to Glx (Bol et al., 2002; Chikaraishi et al., 2007; Gauthier et al., 2013; Hare et al., 1991; Hofmann et al., 1995; McClelland and Montoya, 2002; Ostle et al., 1999; Smallwood et al., 2003).

2.2. Cereal rachis amino acid δ^{15}N values

Fig. 3 shows the δ^{15}N values of cereal rachis AAs, normalised to Glx, from experimental farming plots at Rothamsted and Bad Lauchstädt. The δ^{15}N values of Phe (+8.2 ± 1.9‰) and Pro (+2.1 ± 1.2‰), are higher than the $\delta^{15}N_{Glx}$ value. The δ^{15}N values of Leu (−1.9 ± 1.9‰), Gly (−4.5 ± 2.0‰) and Ser (−5.9 ± 1.2‰) are lower. Ala, Asx and Val have δ^{15}N values within ±0.6‰ of the $\delta^{15}N_{Glx}$ value. The variation in relative AA δ^{15}N values of cereal rachis is much greater than that of cereal grains.

Fig. 4 relates determined rachis AA δ^{15}N values to known pathways of AA metabolism in whole cereal plants. This indicates the similarity in AA biosynthetic relationships between whole plants and developing cereal grains (see Fig. 2). The relative AA δ^{15}N values in cereal rachis vary considerably more than those in cereal grains. This could reflect the greater potential for input of pre-biosynthesised AAs with different δ^{15}N values to the rachis cells, whereas in developing grains, Gln and Asn are the main source of imported N, which is then used to feed the biosynthesis of other AAs. The δ^{15}N value of Phe in cereal rachis is on average +1.4 ± 0.8‰ greater than that of the Phe in its corresponding cereal grains. This ^{15}N-enrichment could indicate the greater significance

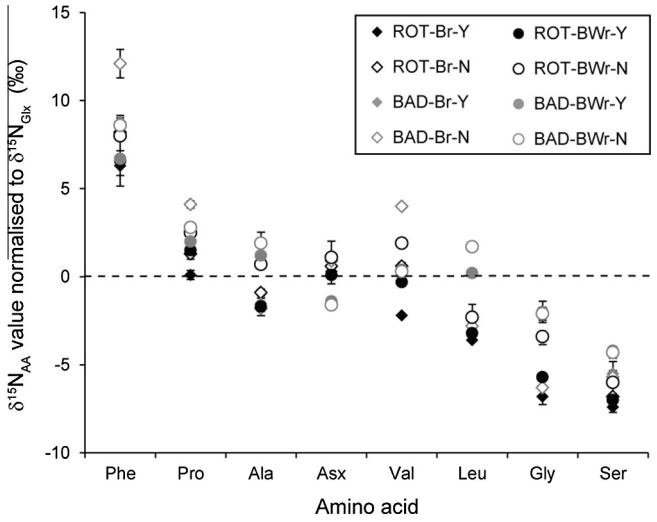

Fig. 3. Amino acid δ^{15}N values normalised to Glx from cereal rachis grown on experimental plots at Rothamsted, UK, and Bad Lauchstädt, Germany. Error bars represent the standard deviation associated with triplicate isotope determinations. Br = barley rachis; BWr = bread wheat rachis.

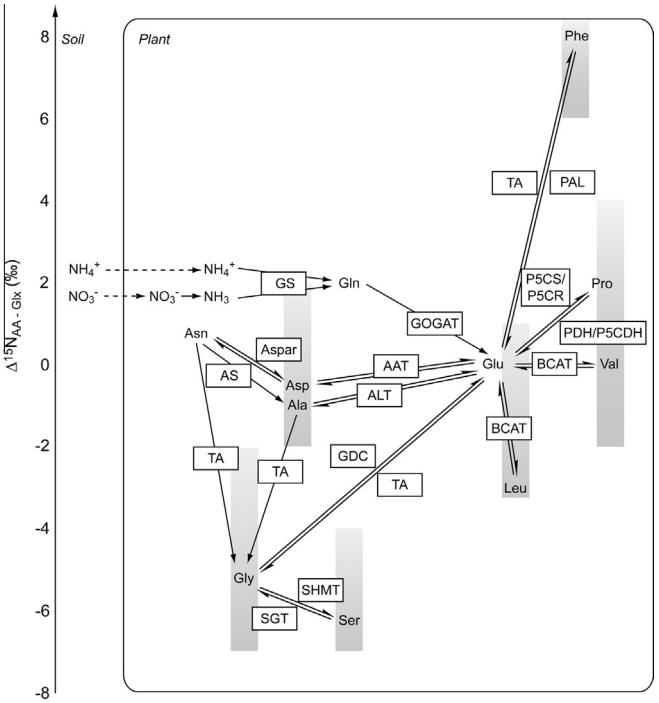

Fig. 4. The relationship between determined cereal rachis AA δ^{15}N values and known pathways of AA metabolism in whole cereal plants. Variation in AA δ^{15}N values is indicated by grey bars. Enzymes are shown in boxes: AAT, aspartate transaminase; ALT, alanine transaminase; AS, asparagine synthetase; Aspar, asparaginase; BCAT, branched-chain aminotransferase; GDC, glycine decarboxylase; GOGAT, glutamate synthase; GS, glutamine synthetase; PAL, phenylalanine:ammonia lyase; P5CS, Δ^1-pyrroline-5-carboxylate synthetase; P5CR, Δ^1-pyrroline-5-carboxylate reductase; PDH proline dehydrogenase; P5CDH, Δ^1-pyrrolidine-5-carboxylate dehydrogenase; SGT, serine:glyoxylate aminotransferase; SHMT, serine hydroxymethyltransferase; TA, transaminases.

of the phenylpropanoid pathway in the metabolism of Phe in rachis cells compared to cereal grains, since rachis is comprised of cells that fulfill a predominantly structural role and are therefore likely to be highly lignified. Thus, it is expected that rachis cells will experience a high turnover of Phe as a substrate in the phenylpropanoid pathway, which leads to the formation of lignin.

2.3. Pulse amino acid δ^{15}N values

Fig. 5 shows the δ^{15}N values of AAs, normalised to Glx, from broad beans and peas grown on plots at Bad Lauchstädt. In broad beans, the δ^{15}N values of Pro (+2.6 ± 1.1‰) and Asx (+1.0 ± 0.6‰) are higher than the $\delta^{15}N_{Glx}$ value, whereas the δ^{15}N values of Ala (−1.5 ± 0.5‰), Leu (−4.5 ± 1.6‰) and Ser (−3.7 ± 0.4‰) are lower. The δ^{15}N values of Phe, Val and Gly are within ±1.4‰ of the $\delta^{15}N_{Glx}$ value. In peas, the δ^{15}N values of Pro (+2.9 ± 0.0‰) and Gly (+6.1 ± 0.4‰) are higher than the $\delta^{15}N_{Glx}$ value, whereas the δ^{15}N value of Leu (−4.6 ± 1.2‰) is lower. The δ^{15}N values of the other AAs, Phe, Ala, Asx, Val and Ser, are within ±1.4‰ of the $\delta^{15}N_{Glx}$ value. The most striking difference between the AA δ^{15}N values of broad beans and peas is the higher δ^{15}N value of Gly observed in peas.

N remobilised from senescing leaves is also the main contributor to legume seed (pulse) N (Murray and Cordova-Edwards, 1984). In legumes, Asn is the major form of translocated N (Miflin and Lea, 1977). N also reaches the seeds in the form of ureides: allantoin and allantoic acid (Peoples et al., 1985). These nitrogenous compounds enter the seed coat and are transformed, since Ala and Gln are the principal nitrogenous solutes released by the empty seed coats of pea and broad bean (Wolswinkel and De Ruiter,

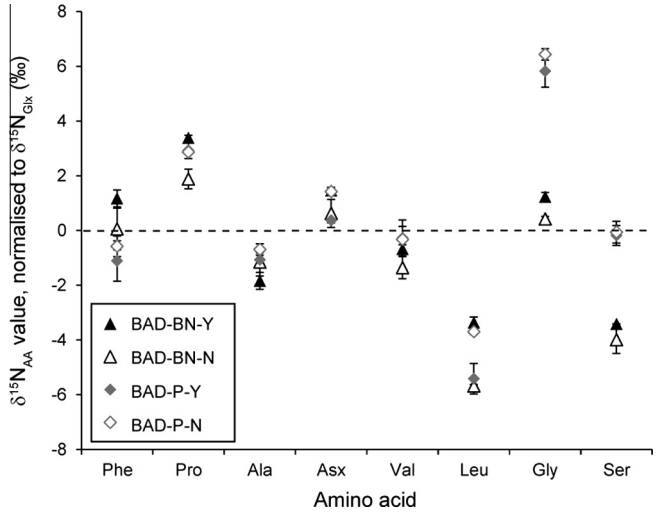

Fig. 5. Amino acid $\delta^{15}N$ values normalised to Glx of broad beans and peas grown on experimental plots at Bad Lauchstädt, Germany. Error bars represent the standard deviation associated with triplicate isotope determinations.

1985). The enzyme asparaginase has been found at high concentrations in the seed coats of peas (Murray and Kennedy, 1980), suggesting Asn that reaches the seed coats is converted to Ala and Gln. The relative ^{15}N-enrichment of Asx compared to the majority of the other AAs in pulses would indicate that deamidation of Asn and transamination of Asp, with associated kinetic isotope effects discriminating against ^{15}N, play a significant role in the biosynthesis of the other AAs in pulses. Pro in broad beans and Pro and Gly in peas are the only AAs ^{15}N-enriched compared to Asx. This is of note, since Pro is not detected in pea or broad bean seed coat exudate, which means it needs to be biosynthesised *in situ* in the seeds (Wolswinkel and De Ruiter, 1985). Gly is not detected in the seed coat exudate of broad bean, but is in peas. The difference between the relative $\delta^{15}N$ values of Gly in peas and broad beans could be due to differences in the proportion of its *in situ* biosynthesis. With the lack of comparable studies citing pulse AA $\delta^{15}N$ values, it is very difficult to explain the observed AA $\delta^{15}N$ values.

2.4. Effect of species on amino acid $\delta^{15}N$ values

Fig. 6 compares the $\delta^{15}N$ values of barley and bread wheat grain, barley and bread wheat rachis and broad bean and pea AAs from the same manuring regimes and the same sites. This allows identification of species-specific differences, eliminating other possible causes of variation. The slope is close to 1 ($y \geqslant 0.94x$) for all of the barley–bread wheat grain pairs, indicating that barley and bread wheat grains have similar relative AA $\delta^{15}N$ values. There is excellent correlation ($r \geqslant 0.98$; $P < 0.01$) between barley and bread wheat grain AA $\delta^{15}N$ values from the same sites and manuring regimes.

The similarity in the relative AA $\delta^{15}N$ values of bread wheat and barley grains (Fig. 6a and b), indicating their similarity in N routing, is expected since both are cereals and would be anticipated to have similar metabolic pathways and uptake mechanisms. These species are closely related – both belonging to the tribe *Triticeae* – and barley is considered a good genomic model for bread wheat (Schulte et al., 2009). Barley and bread wheat can therefore be expected to have the same enzymes and similar isotopic fractionations associated with their AA metabolism in grains.

The $\delta^{15}N$ values of barley and bread wheat rachis AAs from the same manuring regimes and the same sites do not show as strong a

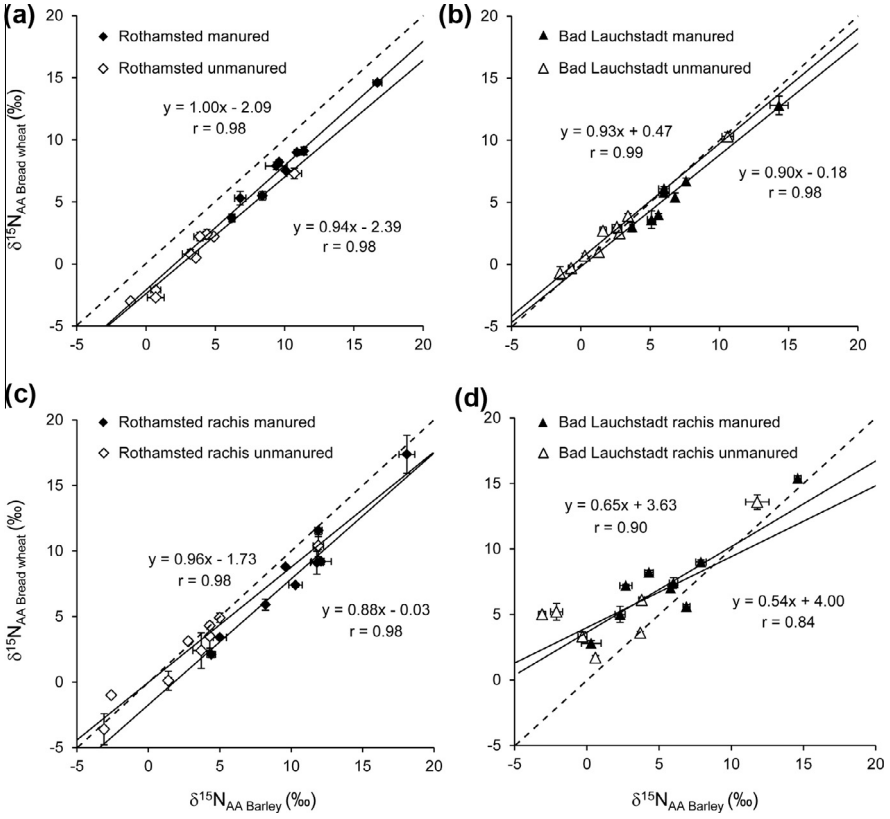

Fig. 6. Correlation between barley and bread wheat AA $\delta^{15}N$ values from: (a) Rothamsted cereal grains, (b) Bad Lauchstädt cereal grains, (c) Rothamsted rachis, and (d) Bad Lauchstädt rachis. Error bars represent the standard deviation associated with triplicate isotope determinations. Dashed line is $y = x$.

correlation as observed with the cereal grains (Fig. 6c and d). The graph comparing barley and bread wheat rachis grown at Rothamsted has a slope close to 1 ($y \geqslant 0.88x$), indicating that barley and bread wheat rachis have similar relative AA δ^{15}N values at Rothamsted. There is also a strong correlation ($r \geqslant 0.98$; $P < 0.01$), which shows that all of the AAs have similar relative δ^{15}N values in these two species. However, the graphs comparing barley and bread wheat rachis grown at Bad Lauchstädt have a mean slope of 0.60 ± 0.08 and exhibit a comparatively weaker correlation ($r \geqslant 0.84$; $P < 0.05$).

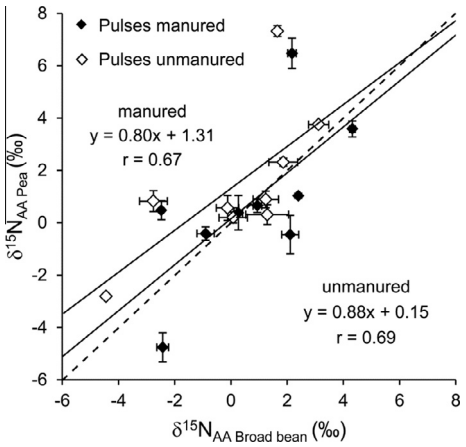

Fig. 7. Correlation between broad bean and pea AA δ^{15}N values. Error bars represent the standard deviation associated with triplicate isotope determinations. Dashed line is $y = x$.

The greater variability in the relative AA δ^{15}N values in barley and bread wheat rachis could indicate that rachis cells are more sensitive to slight differences in growing conditions and adaptations to environment. It is unclear whether the differences in relative AA δ^{15}N values observed in barley and bread wheat rachis grown at Bad Lauchstädt are due purely to species-specific differences or differences in growing conditions between individual plots. It could also reflect inherent variation between plants. The fact that there was a better correlation and a smaller offset between rachis AA δ^{15}N values from barley and bread wheat grown at Rothamsted suggests that the variation in relative AA δ^{15}N values is due to differences in growing conditions rather than species.

The δ^{15}N values of broad bean and pea AAs from the same manuring regimes are compared in Fig. 7. The graphs comparing broad bean and pea AA δ^{15}N values have a mean slope of 0.84 ± 0.06 and a weak correlation ($r \geqslant 0.67$; $P \leqslant 0.05$). The clear difference between relative AA δ^{15}N values in broad beans and peas indicate metabolic differences between the two species, in particular with regards to Gly, despite the fact that they are both leguminous plants.

2.5. Inter-site variation of amino acid δ^{15}N values

Fig. 8 compares the δ^{15}N values of cereal grain and rachis AAs grown at Rothamsted and Bad Lauchstädt. Cereal grains and rachis from the same species and manuring regimes are compared so that any differences between the sites are the only cause of any

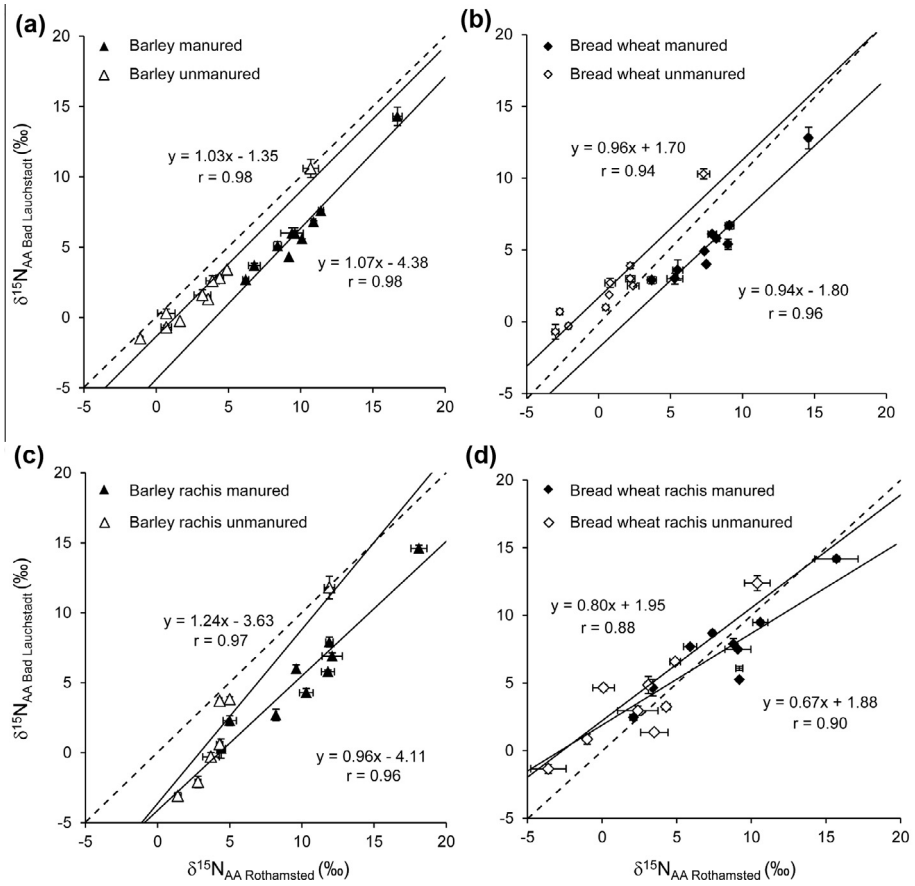

Fig. 8. Correlation between AA δ^{15}N values of crops grown at Rothamsted and Bad Lauchstädt, from: (a) barley grains, (b) bread wheat grains, (c) barley rachis, and (d) bread wheat rachis. Error bars represent the standard deviation associated with triplicate isotope determinations. Dashed line is $y = x$.

variation. The graphs comparing barley grain AA $\delta^{15}N$ values grown at Rothamsted and Bad Lauchstädt (Fig. 8a) have a mean slope of 1.05 ± 0.03 and a very strong correlation ($r \geqslant 0.98$; $P < 0.01$). The graphs comparing bread wheat grain AA $\delta^{15}N$ values at Rothamsted and Bad Lauchstädt (Fig. 8b) have a mean slope of 0.95 ± 0.01 and a strong correlation ($r \geqslant 0.94$; $P < 0.01$).

The lack of significant differences between the relative AA $\delta^{15}N$ values of barley and bread wheat grains grown at Rothamsted and Bad Lauchstädt indicates that differences in climate, rainfall, soil type and growing conditions between the two sites do not have a significant effect on the cycling of AA–N into these cereal grains. Rothamsted has much higher annual rainfall than Bad Lauchstädt (727 mm compared to 483 mm) and the soil types (chromic luvisol and haplic chernozem) at the two sites are different. Thus, there exists potential for differences in growing conditions, notably water availability, at the two sites but this has not influenced the relative AA $\delta^{15}N$ values in cereal grains. This indicates that differences in the growing conditions and climate at Rothamsted and Bad Lauchstädt do not result in significant differences in the relative $\delta^{15}N$ values of AAs in barley and bread wheat grains.

The AA $\delta^{15}N$ values of rachis grown at Rothamsted and Bad Lauchstädt are more variable. Fig. 8c shows a graph comparing manured barley rachis AA $\delta^{15}N$ values grown at Rothamsted and Bad Lauchstädt, which has a slope of 0.96 and a strong correlation ($r = 0.96$; $P < 0.01$). However, the graph comparing unmanured barley rachis grown at Rothamsted and Bad Lauchstädt has a slope of 1.24, indicating that there are site-specific differences between the relative AA $\delta^{15}N$ values in unmanured barley rachis. The graphs (Fig. 8d) comparing bread wheat rachis grown at Rothamsted and Bad Lauchstädt have a mean slope of 0.75 ± 0.09 and a comparatively weaker correlation ($r \geqslant 0.89$; $P < 0.02$).

The difference in relative AA $\delta^{15}N$ values in barley and bread wheat rachis between Rothamsted and Bad Lauchstädt suggests that the cycling of N into rachis cells could be affected by differences in rainfall and soil type. It can be expected that rachis AA metabolism is more sensitive to growing conditions than that of grains since grain development occurs over a short period of time and the turnover of N is relatively small.

3. Conclusions

This study is the first to investigate the $\delta^{15}N$ values of proteinaceous AAs from cereal grains, rachis and pulses grown under controlled conditions. It has been shown that there are appreciable differences in the $\delta^{15}N$ values of individual AA in cereal grains, rachis and pulses, which may arise from isotopic fractionation associated with the metabolic pathways involved in their biosynthesis and catabolism. The relative differences in AA $\delta^{15}N$ values in barley and bread wheat grains and rachis and in broad bean and pea seeds may also provide an insight into the cycling of N in different plant parts and in N_2-fixing and non N_2-fixing plants from different sites within the agricultural ecosystem. A greater understanding of the underlying metabolic pathways influencing bulk plant protein $\delta^{15}N$ values can aid understanding of plant N cycling and differences in the source of assimilated N. The most significant findings were:

Appreciable differences in the $\delta^{15}N$ values of individual cereal grain and rachis AAs, which could be attributed to the metabolic pathways involved in their biosynthesis and catabolism. The relative ^{15}N-enrichment of Phe seems to be due to its involvement in the phenylpropanoid pathway and Glx has a $\delta^{15}N$ value which is an average of the other AAs due to its central role in AA–N cycling.

Barley and bread wheat grains had similar relative AA $\delta^{15}N$ values, indicating a potential similarity in the routing of N into developing barley and bread wheat grains. There was more variability between the AA $\delta^{15}N$ values in rachis than in cereal grains, likely due to the greater potential for cycling of AAs in whole plants, compared to during grain development.

Broad beans and peas had very different relative AA $\delta^{15}N$ values, suggesting differences in the metabolic routing of AAs to the developing seeds in these leguminous plants.

Differences in soil type and rainfall between Rothamsted and Bad Lauchstädt did not affect the relative AA $\delta^{15}N$ values in barley and bread wheat grains, but did in barley and bread wheat rachis, suggesting that rachis AA $\delta^{15}N$ values are more sensitive to differences in growing conditions.

4. Experimental

4.1. Sampling methods

Sampling took place immediately before the harvest, when the crops were fully ripe. First, 30 cereal or pulse plants were harvested along a central transect through each experimental plot. Second, 10 cereal ears, or pulse pods, were randomly chosen from the group of 30 plants. The grains from one side of each of the 10 cereal ears, or half the contents of the pulse pods, were removed for final sampling; all of the rachis from the 10 cereal ears was also taken for analysis. Therefore, each bulk sample represents a pooled sub-sample from 10 different plants from each plot or field (for cereals c. 200–300 grains and for pulses c. 30–50 seeds). For cereals, the grain and rachis in these sub-samples were separated from each other and analysed separately. Samples were ground as single samples to a fine homogeneous powder (<250 μm size) under liquid N using a Spex 6850 freezer mill.

4.2. Bulk $\delta^{15}N$ isotope analysis

Bulk $^{15}N/^{14}N$ analysis was performed by sample combustion in a Flash 112 elemental analyser (ThermoQuest, Milan) linked under continuous flow with a Delta + XL mass spectrometer (ThermoFinnigan, Bremen). Isotope ratios were calculated as $\delta^{15}N$ versus atmospheric N_2 by comparison with standards calibrated against IAEA-N-1 and N-2. The precision (1σ) among replicates of a homogenised barley sample was 0.4‰ for $\delta^{15}N$ analysed in 29 separate runs.

4.3. Preparation of amino acid derivatives (N-acetyl-i-propyl esters)

Lipids were extracted from the powdered samples with chloroform/methanol (2:1 v/v, 10 mL) by ultrasonication. To obtain individual AAs, lipid extracted grain was hydrolysed in culture tubes (6 M HCl, 2 mL, 100 °C, 24 h). The samples were evacuated and heated for 24 h at 100 °C. The solutions were blown to dryness under N_2 and redissolved in 0.1 M HCl and stored at −18 °C until required for analysis. A known quantity of norleucine (1 mg mL^{-1} in 0.1 M HCl) was added to each sample as an internal standard.

Dowex 50WX8, 200–400 mesh ion-exchange resin (Acros Organics) has to be prepared so as to ensure that all cation exchange sites are occupied by H^+ ions. This was achieved by soaking overnight in 3 M NaOH, followed by washing in double-distilled water (DDW) and soaking overnight in 6 M HCl. After washing with DDW, it was pipetted into a flash column to the level of 1 mL DDW. A fraction of the hydrolysed plant sample was added to the column and salts were eluted with 6 mL DDW. The AAs were then eluted with ammonium hydroxide (2 M, 6 ml) and dried under N_2 before undergoing derivatisation.

AAs were converted to their *i*-propyl esters by addition of 1 mL of a 4:1 mixture of isopropanol and acetyl chloride (acetyl chloride added dropwise in an ice bath). Culture tubes were then sealed and

heated at 100 °C for 1 h. Reagents were evaporated under a gentle stream of N_2 at room temperature. Dichloromethane (DCM) was added and evaporated in an ice bath to remove excess reagents. AA *i*-propyl esters were then treated with 1 mL of a mixture of acetic anhydride, triethylamine and acetone (1:2:5, v/v/v; 10 min, 60 °C). Reagents were evaporated under a gentle stream of N_2 in an ice bath. The samples were dissolved in 2 mL ethyl acetate and 1 mL saturated NaCl solution was added. After phase separation, the organic phase was collected and the extraction repeated with an additional 1 mL of ethyl acetate. The combined organic phases were evaporated under N_2 in an ice bath and the residual water removed with successive 1 mL aliquots of DCM and evaporated under N_2 in an ice bath. The *N*-acetyl-*i*-propyl (NAIP) esters were dissolved in ethyl acetate and stored at −18 °C until required for analysis.

4.4. Instrumental analyses

AAs were identified by GC-FID by comparison of their retention times with those of AA standards and their $\delta^{15}N$ values were determined by GC–C–IRMS. A ThermoFinnigan Delta^{Plus} XP system (Thermo Electron Corporation) was used to determine the $\delta^{15}N$ values of derivatised AAs. The mass spectrometer (EI, 100 eV, three Faraday cup collectors *m/z* 28, 29 and 30) was interfaced to a Thermo Electron Trace 2000 gas chromatograph via a ThermoElectron gas chromatograph combustion III interface (CuO/NiO/Pt oxidation reactor maintained at 980 °C and reduction reactor of Cu wire maintained at 650 °C). Samples were introduced using a PTV injector held at 200 °C. Helium at a flow of 1.4 mL min^{-1} was used as the carrier gas and the mass spectrometer source pressure was maintained at 9×10^{-4} Pa. The separation of the AAs was accomplished using a DB-35 capillary column (30 m × 0.32 mm internal diameter; 0.5 μm film thickness; Agilent Technologies, UK). The oven temperature of the GC was started at 40 °C and held for 5 min before heating at 15 °C min^{-1} to 120 °C, then 3 °C min^{-1} to 180 °C, then 1.5 °C min^{-1} to 210 °C and finally 5 °C min^{-1} to 270 °C and held for 1 min. A nafion membrane removed water and a cryogenic trap was employed in order to remove CO_2 from the oxidised and reduced sample.

All $\delta^{15}N$ values are reported relative to reference N_2 of known nitrogen isotopic composition, previously calibrated against the AIR international isotope standard, introduced directly into the ion source in four pulses at the beginning and end of each run. Each reported value is a mean of triplicate $\delta^{15}N$ determinations. An AA standard mixture, comprising AA whose $\delta^{15}N$ values were individually determined by EA-IRMS, was run every three runs in order to monitor instrument performance.

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