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Concentrations and nitrogen isotope compositions of free amino acids in *Pinus massoniana* (Lamb.) needles of different ages as indicators of atmospheric nitrogen pollution

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HIGHLIGHTS

- Age-related free amino acid (FAA) $\delta^{15}\text{N}$ variations in needles were first reported.
- FAAs in needles are sensitive biomarkers for atmospheric N pollution.
- FAA $\delta^{15}\text{N}$ variations in needles reflect the main N sources.

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ABSTRACT

Free amino acid $\delta^{15}\text{N}$ values and concentrations of current-year new (new), current-year mature (middle-age) and previous-year (old) *Pinus massoniana* (Lamb.) needles were determined for five sites with different distances from a highway in a forest in Guiyang (SW China). Needle free amino acid concentrations decreased with increasing distance from the highway, and only the free amino acid concentrations (total free amino acid, arginine, γ -aminobutyric acid, valine, alanine and proline) in the middle-aged needles demonstrated a strong correlation with distance from the highway, indicating that free amino acid concentrations in middle-aged needles may be a more suitable indicator of nitrogen (N) deposition compared to new and old needles. Needle free amino acid $\delta^{15}\text{N}$ values were more positive near the highway compared to the more distant sites and increased with increasing needle age, indicating that N deposition in this site may be dominated by isotopically heavy $\text{NO}_x\text{-N}$ from traffic emissions. In sites beyond 400 m from the highway, the $\delta^{15}\text{N}$ values of total free amino acids, histidine, glutamine, proline, alanine, aspartate, isoleucine, lysine, arginine and serine in each age of needle were noticeably negative compared to their respective $\delta^{15}\text{N}$ values near the highway. This suggested that needle free amino acid $\delta^{15}\text{N}$ values from these sites were more affected by ^{15}N -depleted atmospheric $\text{NH}_x\text{-N}$ from soil emissions. This result was further supported by the similarity in the negative moss $\delta^{15}\text{N}$ values at these sites to the $\delta^{15}\text{N}$ values of soil-derived $\text{NH}_x\text{-N}$. Needle free amino acid $\delta^{15}\text{N}$ values therefore have the potential to provide information about atmospheric N sources. We conclude that needle free amino acid concentrations are sensitive indicators of N deposition and that the age-related free amino acid $\delta^{15}\text{N}$ values in needles can efficiently reflect atmospheric N sources. This would probably promote the application of the combined plant tissue amino acid concentration and $\delta^{15}\text{N}$ analyses in N deposition bio-monitoring.

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1. Introduction

Dramatically elevated atmospheric N deposition is an increasing global problem, particularly in many regions of Asia that are associated with rapid industrialization and urbanization (Kim and Cho, 2003), and the detrimental impacts of the heavy atmospheric

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reactive N input on natural ecosystems and air quality have been widely reported (Krupa, 2003; Stevens et al., 2006; Skinner et al., 2006; Zamboni et al., 2009). To efficiently control the levels of atmospheric N pollution and further study environmental effects of N deposition, their sources and distribution must be investigated. However, the wide range of nitrogenous compounds that exist in precipitation, aerosols and the gas phase have made the systematically instrumental monitoring of N deposition highly complex and costly. Bio-monitors, by contrast, may provide an alternative for evaluating atmospheric N pollution.

The significant relationship between plant tissue (both mosses and vascular plants) N concentrations and atmospheric N deposition has been frequently found in field study (Hicks et al., 2000; Pitcairn et al., 2001, 2003). Thus, the analysis of plant tissue N concentrations can be applied effectively in evaluating the level of atmospheric N deposition. Proteins are fundamental building blocks of life that are composed of amino acids (biological N-containing compound). The ability of plants to respond to various environmental stresses (e.g., salt, pest damage, pathogens, heat, cold, water stress and metal) with changes in the composition and size of the tissue amino acid pool is widely recognized (Sagisaka, 1974; Di Martino et al., 2003; Pavlíková et al., 2014; Hu et al., 2015). Because plant leaves can directly absorb atmospheric organic and inorganic N compounds and rapidly convert them to useful amino acids (e.g., glutamate and aspartate) (Geßler et al., 2002; Yoneyama et al., 2003; Murphy et al., 2006; Lockwood et al., 2008), an extra N supply can also cause a significant increase in the concentrations of leaf free amino acids (Ferm et al., 1990; Pietilä et al., 1991). Moreover, the accumulation of leaf amino acid N in response to N inputs is mainly the result of increasing asparagine, glutamine and arginine (Nordin et al., 1998). Several studies have also suggested that free amino acids in plant leaves are useful biomarkers for atmospheric N pollution (Huhn and Schulz, 1996; Pitcairn et al., 2003). Therefore, free amino acid concentrations in plant leaves are able to indicate the varying N deposition to some extent.

The $\delta^{15}\text{N}$ values of plant tissues are often considered to be indicators that reflect the effects of a range of environmental processes (Durka et al., 1994; Evans, 2001; Dawson et al., 2002). Mosses depend on atmospheric N inputs as their main N source, and because isotopic fractionation during the uptake of N by mosses is assumed to be insignificant (Bragazza et al., 2005), it is generally accepted that moss $\delta^{15}\text{N}$ values can be used to indicate the prevailing N emission sources (Pearson et al., 2000; Skinner et al., 2006). In contrast, foliar $\delta^{15}\text{N}$ values of vascular plants may be an integrated result of many factors. These factors include atmospheric N, soil N availability, root depth, plant mycorrhizal status, isotopic fractionation during N transportation, aridity, and anthropogenic N addition (Högberg, 1997; Evans, 2001; Choi et al., 2002; Asada et al., 2005). Although there is uncertainty about using vascular plant foliar $\delta^{15}\text{N}$ values to detect signals of atmospheric N pollution in natural habitats, changes in plant foliar $\delta^{15}\text{N}$ values are frequently observed following exposure to nitrogenous pollutants. For example, Xiao et al. (2011) reported that camphor leaf $\delta^{15}\text{N}$ values effectively reflect atmospheric NO_x -N sources from traffic emissions and atmospheric NH_3 sources from chemical fertilizer plant emissions, with more positive leaf $\delta^{15}\text{N}$ values (up to +2.5‰) near roads and strongly ^{15}N -depleted leaves (as low as -11.8‰) near a chemical fertilizer plant. Ammann et al. (1999) distinguished differences between the $\delta^{15}\text{N}$ values in *Picea abies* needles that were collected near a motorway (up to +2‰) and far from it (as low as -4.4‰). In addition, several studies even have suggested that tree ring $\delta^{15}\text{N}$ values can respond to different atmospheric N sources (Savard et al., 2009; Kwak et al., 2009; Sun et al., 2010). However, few studies have addressed the bio-responses of N

deposition at the molecular level. In fact, the determination of plant tissue amino acid $\delta^{15}\text{N}$ values can be used as a novel approach for evaluating environmental and anthropogenic effects on plant metabolism and for understanding the responses of plants to environmental stresses (Molero et al., 2011; Gauthier et al., 2013; Styring et al., 2014a). In recent years, considerable advances have been made towards understanding amino acid $\delta^{15}\text{N}$ values in plant tissues, and to date, studies of amino acid $\delta^{15}\text{N}$ values in plant tissues mainly concentrated on: (1) estimating the trophic levels of organisms in food webs (Chikaraishi et al., 2009); (2) reconstructing human palaeodiet (Styring et al., 2015); (3) examining the effects of land use and discriminating between different plant species associated with the acquisition of available N sources (Bol et al., 1998, 2002); (4) investigating the ecotoxicological effects of ozone on plants (Hofmann et al., 1995); (5) understanding leaf metabolic fluxes (Gauthier et al., 2013) and (6) revealing the effect of manure on N metabolism in different crop species and plant parts (Styring et al., 2014b). By comparison, studies on the use of foliar free amino acid $\delta^{15}\text{N}$ values of vascular plants as indicators of atmospheric N sources in the natural environment are absent. And more importantly, tissue amino acids are the most key targets for plant N metabolism. Thus, verification of the ability to use the free amino acid $\delta^{15}\text{N}$ values in plant leaves to identify the sources of atmospheric N pollution is important and meaningful. ^{15}N labeling experiments have indicated that the ^{15}N enrichment of sunflower leaf amino acids (e.g., glutamine, proline, alanine, serine, glycine, glutamic and aspartic acid) increased with increasing atmospheric $^{15}\text{NO}_2$ concentration (Segschneider et al., 1995). A study by Paolini et al. (2015) also suggested that free amino acids are indeed the prevailing low molecular weight biological N-containing molecules in plant tissue, and their $\delta^{15}\text{N}$ patterns record useful information about the growth environment. As mentioned above, the elevated N deposition can lead to a significant increase in the concentrations of leaf free amino acids. This implies that ^{15}N abundances in individual leaf free amino acids may be closely related to atmospheric N pollution. Therefore, we expect that nitrogen isotope compositions of free amino acids in leaves can be used as more sensitive indicators of atmospheric N pollution than the $\delta^{15}\text{N}$ values of bulk leaf N.

Free amino acid N isotopic compositions in plant tissues are potentially useful tools for providing very specific information about biogeochemical, environmental, and ecological processes (Hofmann et al., 1997; Fraser et al., 2011; Styring et al., 2015). Therefore, studies on the foliar free amino acid ^{15}N abundance could also be helpful for our understanding of the variation in leaf amino acid metabolic fluxes under condition of high N inputs, probably driving a further application of plant amino acid $\delta^{15}\text{N}$ analysis in N deposition bio-monitoring. In this study, Masson pine trees (*Pinus massoniana* Lamb.), which are widely distributed in Guiyang city, were selected to determine if their leaf free amino acid $\delta^{15}\text{N}$ values can effectively indicate atmospheric N sources. On the basis of the above statement, the following questions will be discussed further: (1) Which free amino acids in needles are involved in the response to the atmospheric N pollution? (2) How do the foliar free amino acid concentrations and $\delta^{15}\text{N}$ values vary with age? and (3) Can the free amino acid $\delta^{15}\text{N}$ values in Masson pine needles of different ages be used to identify atmospheric N sources?

2. Materials and methods

2.1. Study area

The study was conducted in a mixed pine and broadleaf forest (Lu Chong Guan Forest Park, 26°38' N, 106°43' E) in Guiyang city

(SW China). The study area is characterized by a subtropical monsoon climate with a southeast prevailing wind direction in the summer and northeast wind all year. The average altitude is 1250 m above sea level. The annual mean temperature is 15.3 °C (annual mean relative humidity of 86%) and the precipitation amount is 1,175 mm. The soil is acidic and strongly weathered (yellow soil in the Chinese classification system) with low base saturation. Samples were collected along a distance gradient to a highway that crosses the forest in a northeast direction and has a traffic density of approximately 12,700 vehicles per day in the year 2015. The sites were 4 m (S1), 15 m (S2), 50 m (S3), 400 m (S4) and 800 m (S5) from the highway in the southeast direction (the prevailing wind direction). The average atmospheric NO₂ concentration in 2015 was 42.3 µg m⁻³ close to the highway, which decreased to 9.5 µg m⁻³ at a distance of 800 m, and the average total N deposition in this study area has been estimated to be approximately 22.8 kg N ha⁻¹ yr⁻¹ (Xiao et al., unpublished data).

2.2. Sample collection and treatment

The sampling season was May 2015. Masson pine trees of similar ages (approximately 22 years) and sizes (approximately 14 m in height) were selected to reduce the influence of canopy height and soil depth of the roots between sampling trees. 4–5 moss samples (mainly *Haplocladium* (*H. microphyllum*) and *Eurohypnum* (*E. leptothallum*)) were collected from the surrounding bare rocks (were not covered by canopies) before the needle samples were collected at each site. Approximately 4–6 g of new current-year leaves (new, verdant needles at the shoot apex), mature current-year leaves (middle-aged, verdant needles that were longer than new needles) and previous-year leaves (old, non-verdant needles grown in the year 2014) were collected from the outer branches in the east, south, north, and west directions (approximately 12 m above the ground). For each type of needle, we collected 5–8 representative samples from 1 to 3 selected trees at each site. Sampling was performed under the weather conditions of no rain, and only healthy samples were collected. 3–4 soil samples (approximately 100 g) were collected from the topmost 10 cm soil layer with a high density of roots after sampling the leaves at each site, and the roots and needle litter were removed immediately.

All fresh samples were put into plastic bags, labeled and stored immediately in an insulated, chilled box. In the laboratory, all washed plant samples were freeze-dried, and all soil samples were dried at 80 °C for 24 h. Finally, all samples were pulverized for homogenization. The plant powder samples were stored in liquid nitrogen before use. The soil samples were preserved in a desiccator.

2.3. Analysis of N concentrations and δ¹⁵N values

N concentration (%; dry weight) analyses were performed using a vario MACRO cube elemental analyzer (Elementar, Frankfurt, Germany). The analytical precision (n = 3) of the N concentrations was better than 0.02%. The δ¹⁵N values of samples and non-derivatized single amino acid standards were determined using an EA/IRMS system (a Thermo MAT253 isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany) connected to a Flash EA 2000 elemental analyzer (Thermo Scientific, Bremen, Germany)). The δ¹⁵N values (analytical precision of 0.1‰; n = 3) were calculated against ammonium sulfate (IAEA-N-1), potassium nitrate (IAEA-NO3) and L-glutamic acid (USGS 40). Each reported N concentration and δ¹⁵N value was a mean of at least triplicate measurements. The isotope ratios were expressed in per mil (‰) relative to atmospheric N₂.

2.4. Reagents and reference materials

Amino acid standards (asparagine (Asn), glutamine (Gln), glutamate (Glu), isoleucine (Ile), leucine (Leu), lysine (Lys), arginine (Arg), aspartate (Asp), serine (Ser), tryptophan (Trp), citrulline (Cit), glycine (Gly), threonine (Thr), ornithine (Orn), alanine (Ala), proline (Pro), methionine (Met), cystine (Cy2), valine (Val), phenylalanine (Phe), histidine (His), α-aminobutyric acid (AABA), γ-aminobutyric acid (GABA), hydroxyproline (Hyp), theanine (Thea), tyrosine (Tyr), norvaline (Nva) and sarcosine (Sar)), pyridine (Py), *N*-methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide (MTBSTFA), trifluoroacetic acid (TFA), anhydrous sodium sulfate (Na₂SO₄) and cation-exchange resin (Dowex 50 W × 8 H⁺, 200–400 mesh size) were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, USA) in the highest purity available. Ultrapure grade water was obtained from a Milli-Q system (Millipore, Billerica, USA) in the laboratory.

2.5. Analysis of amino acid concentrations

A 0.15 g sample of leaf powder was homogenized with 1.8 mL of TFA (10% v/v) (200 µL, 1 nmol µL⁻¹ sarcosine and α-aminobutyric acid added as internal standards) by sonication for 25 min at 4 °C. The homogenate was centrifuged at 14,000 rpm for 10 min at 4 °C, after which the supernatant was collected. A 2 mL TFA (10% v/v) was added into the remaining sample to maximize the recovery of free amino acids from the sample. The supernatants were combined and purified with Amicon Ultra 3K centrifugal filter tubes (Millipore, Billerica, USA). The purified solutions were analyzed using an Agilent 1260 series HPLC system (Agilent Technologies, Palo Alto, USA). The instrumental methods for analysis of the amino acid concentrations are performed as described in Xu and Xiao (2017).

2.6. Analysis of amino acid δ¹⁵N values

A 0.2 g sample of leaf powder was homogenized with 2 mL of TFA (10% v/v) by sonication for 25 min at 4 °C. The homogenate was centrifuged at 14,000 rpm for 10 min at 4 °C and the supernatant was collected. The same extraction procedure was repeated twice. The supernatants were combined and purified with Amicon Ultra 3K centrifugal filter tubes (Millipore, Billerica, USA). Next, 200 µL of α-aminobutyric acid (1 nmol µL⁻¹) was added into the purified supernatant as an internal reference (δ¹⁵N = 8.43 ± 0.03‰). After the purified supernatant (blown under N₂ for 2 h) was freeze-dried, the remaining fraction was resuspended in 1 mL of HCl (0.1 mol L⁻¹) and passed through a cation-exchange column (Dowex 50 W × 8 H⁺, 200–400 mesh size; Sigma-Aldrich). The amino acids were eluted with 8 mL of an NH₄OH aqueous solution (10 wt%). The solution (blown under N₂ for 2 h) was then freeze-dried. All extracts were stored at -80 °C until further analysis. The derivatization and analysis procedures of the amino acids were carried out according to the methods of Molero et al. (2011) and Zhang et al. (2016). In brief, approximately 150 µg of anhydrous Na₂SO₄ were added to the purified samples, and the samples were then derivatized with 50 µL of pyridine and 50 µL of *N*-methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide. The vials containing the samples were incubated at 90 °C for 1 h.

To confirm the structures of the amino acid derivatives and check the chromatographic sequence of the amino acid derivatives, derivatized amino acid standard mixtures (Asn, Gln, Glu, Ile, Leu, Lys, Arg, Asp, Ser, Trp, Gly, Thr, Ala, Pro, Met, Val, Phe, His, AABA and Tyr) were analyzed by GC/MS, which consisted of a GC6890 gas chromatograph (Agilent Technologies, Palo Alto, USA) coupled to a time of flight Mass Spectrometer (GCT) (Waters, Milford, USA). The GC column and conditions employed on the GC/MS were consistent with the GC/C/IRMS analysis described below. Individual amino

acid N isotopic analysis was performed using a Trace GC Ultra (consisting of a GC IsoLink (Thermo Scientific, Bremen, Germany) and a ConFlo IV interface (Thermo Scientific, Bremen, Germany)) interfaced with a MAT253 IRMS (Thermo Scientific, Bremen, Germany). The amino acids were separated on a DB-5 column (30 m × 0.25 mm × 0.25 μm; J&W Scientific, Folsom, USA) with helium as the carrier gas. A volume of 0.5–1.0 μL of each sample (depending on the amino acid concentrations in the samples) was injected in split-less mode at an injector temperature of 270 °C using a triplus autosampler (Thermo Fisher Scientific, Bremen, Germany). To prolong the lifetime of the catalyst in the reactor, the system was back-flushed with helium at the first 15 min. The oven temperature of the GC was started at 90 °C (held for 1 min), then heated at 8 °C min⁻¹ to 140 °C (held for 5 min) before ramping at 3 °C min⁻¹ to 220 °C and finally at 12 °C min⁻¹ to 285 °C (held for 12.5 min). The combustion furnace reactor (combined oxidation and reduction in one unit) was operated at 1,030 °C. Water vapor was removed using a Nafion membrane, and CO₂ was trapped by a liquid N₂ trap.

We found that the resin did not lead to a modification of the amino acid δ¹⁵N values. The first analytical run was used to balance the system and the results were not used for data analysis. The δ¹⁵N value of the α-aminobutyric acid internal standard in each sample was checked to confirm the reproducibility of the isotope measurements. Moreover, a derivatized mixture of amino acid standards was measured after every 3 runs to monitor the instrumental performance and implement subsequent drift corrections. The precision of the δ¹⁵N measurements (n = 6) for derivatized mixture of amino acid standards averaged 0.6‰ and ranged from 0.1‰ to 1.0‰. The δ¹⁵N values of the underderivatized amino acids measured by EA/IRMS were linearly correlated with those of the derivatized amino acids measured by GC/C/IRMS ($R^2 = 0.994$, $P < 0.001$). The difference between amino acid δ¹⁵N values measured by EA/IRMS and by GC/C/IRMS after empirical correction ranged from -0.8‰ to +0.6‰. Each reported value is a mean of at least triplicate δ¹⁵N determinations.

2.7. Data analysis

Pearson's correlations were conducted to examine the relationship between foliar free amino acid concentration and distance from the highway, and the logarithmic regression was analyzed to evaluate changes in the foliar free amino acid concentrations as a function of distance from the highway. One-way ANOVA followed by a Tukey-HSD test was used to examine the differences between groups of samples (the results were accepted at $P < 0.05$). Statistical analyses were carried out in SPSS 19.0 (SPSS Science, Chicago, USA), and all graphs were created using Origin 9.0 (OriginLab Corporation, Massachusetts, USA).

3. Results

3.1. N concentrations and δ¹⁵N values in needles, mosses and soils

The N concentrations in the needles varied from 1.14% to 1.68%

(Table 1) and decreased slightly with increasing needle age (decreases of less than 21% from new to old needles at all sites). Tissue N concentrations of all moss samples ranged from 1.61% to 2.71% (Table 1). A trend of decreasing concentration with increasing distance from the highway was found in the moss N concentrations, with the highest mean determined for the site closest to the highway (Table 1), and this pattern of moss N concentrations can be clearly related to the emission of NO_x from the traffic. The N concentrations in soils ranged between 0.16% and 0.26% (Table 1) with a mean of 0.19 ± 0.03%. The soil N concentrations between sampling sites did not show significant differences ($P > 0.05$).

The δ¹⁵N values of the needles varied widely from -3.5‰ to +6.3‰. The needle δ¹⁵N values showed a strong gradient with increasing distance from the highway, with the more positive δ¹⁵N values close to the highway and the more negative δ¹⁵N values in the most distant site (S5) (Fig. 1). The older needles in sites S1 and S2 were characterized by higher δ¹⁵N values compared to the new needles, whereas in sites greater than 15 m from the highway, the needle δ¹⁵N values decreased with increasing needle age (Fig. 1).

Unlike the large range of δ¹⁵N values in the needles, the soil δ¹⁵N values were limited from +2.6‰ to +6.7‰ and exhibited a relatively weak gradient with increasing distance from the highway compared to the needle δ¹⁵N values (Table 1). Therefore, the difference between the δ¹⁵N values of the soils and needles (especially the old needles) should increase with increasing distance from the highway.

A decreased gradient with increasing distance from the highway was also found in the moss δ¹⁵N values, but the positive δ¹⁵N values were only observed for site S1 (Fig. 1). The pattern in the moss δ¹⁵N values indicated the existence of a pollution gradient with

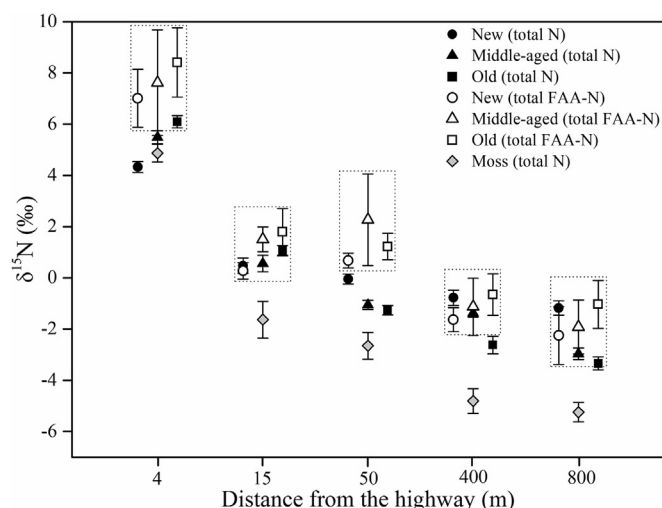


Fig. 1. Variations in δ¹⁵N values and total free amino acid δ¹⁵N values for new, middle-aged and old needles and mosses from five sites at different distances from the highway. The δ¹⁵N values of total free amino acid-N (FAA-N) were calculation based on the concentrations and δ¹⁵N values of all detectable free amino acids. The vertical lines represent standard deviations.

Table 1

The average N concentrations in needles, mosses and soils and the average δ¹⁵N values in soils at five sites with different distances from the highway (minimum and maximum values are given in parentheses).

Distance from the highway	4 m	15 m	50 m	400 m	800 m
Needle N conc. (%)	1.53 (1.27; 1.68)	1.36 (1.21; 1.52)	1.32 (1.18; 1.46)	1.38 (1.20; 1.54)	1.35 (1.14; 1.54)
Moss N conc. (%)	2.49 (2.32; 2.71)	1.87 (1.72; 2.01)	1.71 (1.61; 1.81)	1.80 (1.67; 2.01)	1.72 (1.68; 1.78)
Soil N conc. (%)	0.19 (0.18; 0.22)	0.18 (0.17; 0.20)	0.20 (0.16; 0.25)	0.22 (0.18; 0.26)	0.17 (0.16; 0.21)
Soil δ ¹⁵ N (‰)	+5.8 (+5.2; +6.7)	+4.8 (+4.3; +5.1)	+4.5 (+3.9; +4.8)	+3.2 (+2.6; +4.0)	+3.6 (+2.9; +4.6)

increasing distance from the highway.

3.2. Free amino acid concentrations in needles

The concentrations of Asp, Gln, Try, Glu, Asp, Phe, Arg, Leu, Ser, Gly, Orn, Ala, Pro, Thr, Lys, Ile, Tyr, His, GABA, Cit, Val and Met in the needles were determined. Met, Cit and Orn were only detected in some of the needles with relatively high N concentrations. The patterns of the main amino acid concentrations are displayed in Fig. 2. The concentrations of Arg, Asp, Glu, Gln, Ala, Val, GABA and Pro in new and middle-aged needles, which were relatively higher, accounted for more than 93% of the total free amino acid concentrations. Furthermore, the concentrations of needle free amino acids significantly decreased with increasing needle age for all sites (Fig. 2). The largest variation in Arg concentration was observed in the needles of different ages and from different sites.

The free amino acid concentrations of the needles were higher in the vicinity of the highway than in more distant sites, and a significant decrease was found in total free amino acid, Arg, GABA, Val, Ala and Pro concentrations of the middle-aged needles as a function of distance from the highway ($P < 0.05$) (Fig. 3). This indicated that the considerable changes in the relative concentrations of free amino acids may be related to a variation in the atmospheric N input.

3.3. The $\delta^{15}\text{N}$ values of needle free amino acids

The $\delta^{15}\text{N}$ values of seventeen free amino acids (Phe, Tyr, His, Glu, Gln, Pro, Ala, Val, Leu, Asp, Asn, Thr, Ile, Lys, Arg, Gly and Ser) in the needles were measured using the method described in this study. The average $\delta^{15}\text{N}$ values of each needle free amino acid (averaged all type of needle) showed a significantly declined trend from the sites near the highway to the more distant sites (Fig. 4). We can observe that Glu, the precursor of most other amino acids, plays an important role in amino acid metabolism (Fig. 4). However, the precision of the Glu $\delta^{15}\text{N}$ measurements for few samples was more than 1.0‰, so the data of Glu $\delta^{15}\text{N}$ was not further discussed in this study.

In the site with direct proximity to the highway, a relatively high ^{15}N enrichment in all detectable free amino acids was observed (Fig. 5), and the patterns of their isotope ratio variations from new to old needles were similar to that of the needle $\delta^{15}\text{N}$ values and the total needle free amino acid $\delta^{15}\text{N}$ values (Fig. 1), clearly indicating that this may be caused by N deposition with a high $\delta^{15}\text{N}$. The $\delta^{15}\text{N}$ values of free amino acids in each type of needle at the more distant sites decreased markedly. The $\delta^{15}\text{N}$ values of total free amino acids, Phe, Pro, Val, Leu, Asp, Asn, Lys, Arg, and Ser in each type of needle at site S5 were particularly depleted by 3–11 $\delta^{15}\text{N}\text{‰}$ units compared to their respective $\delta^{15}\text{N}$ values at site S1 (Figs. 1 and 5). However, not all of the detectable free amino acid $\delta^{15}\text{N}$ values in the needles from site S5 exhibited a decreasing trend with increasing needle age. Only His, Gln, Pro, Ala, Asp, Ile, Lys, Arg and Ser had evident ^{15}N depletion, and among these amino acids, only the $\delta^{15}\text{N}$ values of His, Asp, Ile, Lys, Arg and Ser showed a slight decrease from new to old needles (Fig. 5). Interestingly, we observed that the variation pattern of the total free amino acid $\delta^{15}\text{N}$ values from new to old needles in sites beyond 50 m from the highway was contrary to that of the needle $\delta^{15}\text{N}$ values and that the total free amino acid $\delta^{15}\text{N}$ values in the older needles (middle-aged and old) were higher than the corresponding needle $\delta^{15}\text{N}$ values (Fig. 1).

4. Discussion

Excessive N deposition is widely recognized to result in elevated N and free amino acid concentrations in leaves (Baxter et al., 1992;

Van den Berg et al., 2008). For instance, Nordin et al. (1998) reported that free amino acid concentrations in *Deschampsia flexuosa* leaves increased by over 50% following exposure to a simulated N deposition of $50 \text{ kg N ha}^{-1} \text{ yr}^{-1}$, compared to increases of less than 25% in foliar N concentrations. A study by Huhn and Schulz (1996) found that needle free amino acid and N concentrations were relatively low in Neuglobsow (Brandenburg) with an average atmospheric NO_x concentration of $9 \mu\text{g m}^{-3}$. However, in needles collected at Rösa (Sachsen-Anhalt) where the average atmospheric NO_x concentration was $32 \mu\text{g m}^{-3}$, significant accumulation of free amino acid concentrations was observed (especially for Arg, with more than a 150-fold difference), and leaf N concentrations, by contrast, increased slightly. Similarly, in this study, we found that in contrast to a logarithmic decrease in free amino acid concentrations with increasing distance from the highway in middle-aged needles, needle N concentrations only exhibited a slight decrease (less than a 22% difference). It is interesting to note that a larger increasing proportion occurred in foliar free amino acid concentrations (especially for Arg in most cases) in response to increased atmospheric N inputs rather than in the total N concentration of leaves. Considerable amounts of gaseous and aerosological N compounds (e.g., NO , NO_2 , NH_4^+ , NH_3 , HNO_3 and RO_2NO_2) can enter leaves through stomatal uptake and cuticular diffusion. However, the atmospheric N species of interest are generally absorbed into leaves through stomates rather than through the hydrophobic cuticle based on their hydrophilic nature (Bruckner et al., 1993; Calanni et al., 1999). Once these atmospheric N compounds enter leaves, they are rapidly converted to other N forms (e.g., amino acids) (Nussbaum et al., 1993; Yoneyama et al., 2003). Amino acids are preferential N storage and transport compounds (especially Arg with its favorable C/N ratio of 1.5) because of the lower maintenance costs for the use of tissue amino acids N compared to tissue inorganic N (Pate and Layzell, 1990; Huhn and Schulz, 1996). Moreover, because a much more percentage of the total leaf N is contained in proteins, only a relatively small amount exists as free amino acid N (less than 5% in this study) (Näsholm and Ericsson, 1990), more sensitive changes in foliar free amino acid concentrations could be reflected following varied atmospheric N inputs. Interestingly, we also found that the proportional decrease in the foliar free amino acid concentrations (decreases of over 76% for total free amino acid concentrations) from new to old needles was greater than the decrease in the foliar N concentrations (decreases of less than 21%). In theory, older leaves should be able to accumulate more atmospheric N compounds with exposure time, but higher N and free amino acid concentrations in older leaves were not observed in this study. Thus, a possible explanation is that atmospheric N compounds absorbed by older leaves were translocated into other tissues. Since amino acid pools are more readily available for plant metabolism as mentioned above, the decrease in free amino acid concentrations from new to old needles are greater than the decreases in total N concentrations.

The importance of Arg, Asn, Gln, Gly and Phe in foliar free amino acid pools has been demonstrated by previous studies. For instance, a study by Power and Collins (2010) found that free Lue ($R^2 = 0.293$), Ile ($R^2 = 0.336$), Asn ($R^2 = 0.438$), Gly ($R^2 = 0.528$), Gln ($R^2 = 0.661$) and Phe ($R^2 = 0.774$) concentrations in *Calluna* leaves showed a significant response to atmospheric N deposition from an urban-rural gradient in London. For Arg concentrations in plant leaves, strong correlations were found between free Arg concentrations in *Pseudoscleropodium purum* ($R^2 = 0.780$), *Brachythecium rutabulum* ($R^2 = 0.899$) and *Rhytidadelphus triquetrus* ($R^2 = 0.970$) and log distance from a poultry farm (Pitcairn et al., 2003). In this study, free Arg, GABA, Val, Ala, Pro and total free amino acid concentrations in middle-aged needles were significantly correlated with increasing distance from the highway (Fig. 3), which provides

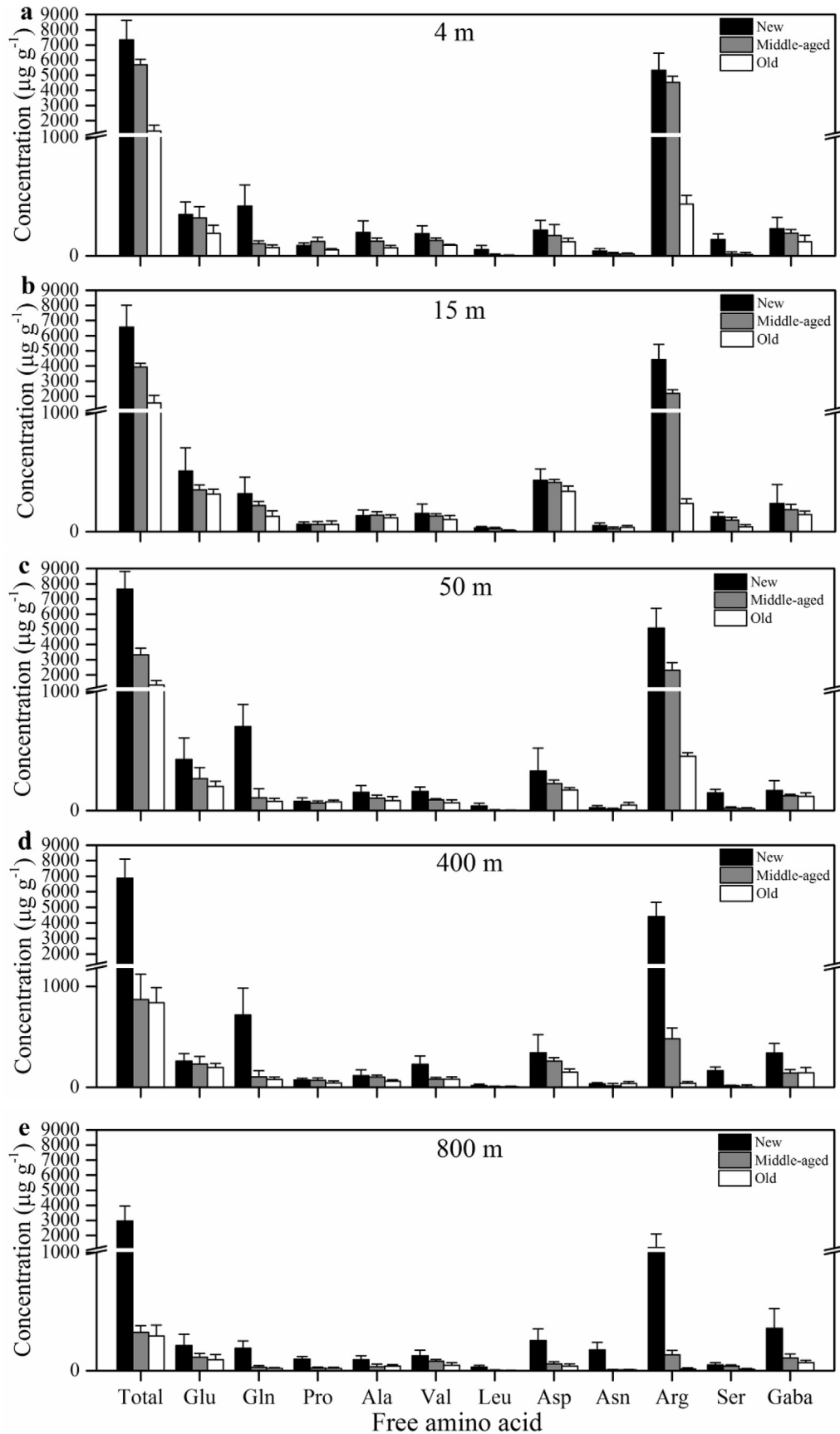


Fig. 2. Concentrations of major free amino acids in needles from five sites at different distances from the highway: a) 4 m, b) 15 m, c) 50 m, d) 400 m and e) 800 m. The vertical lines represent standard deviations.

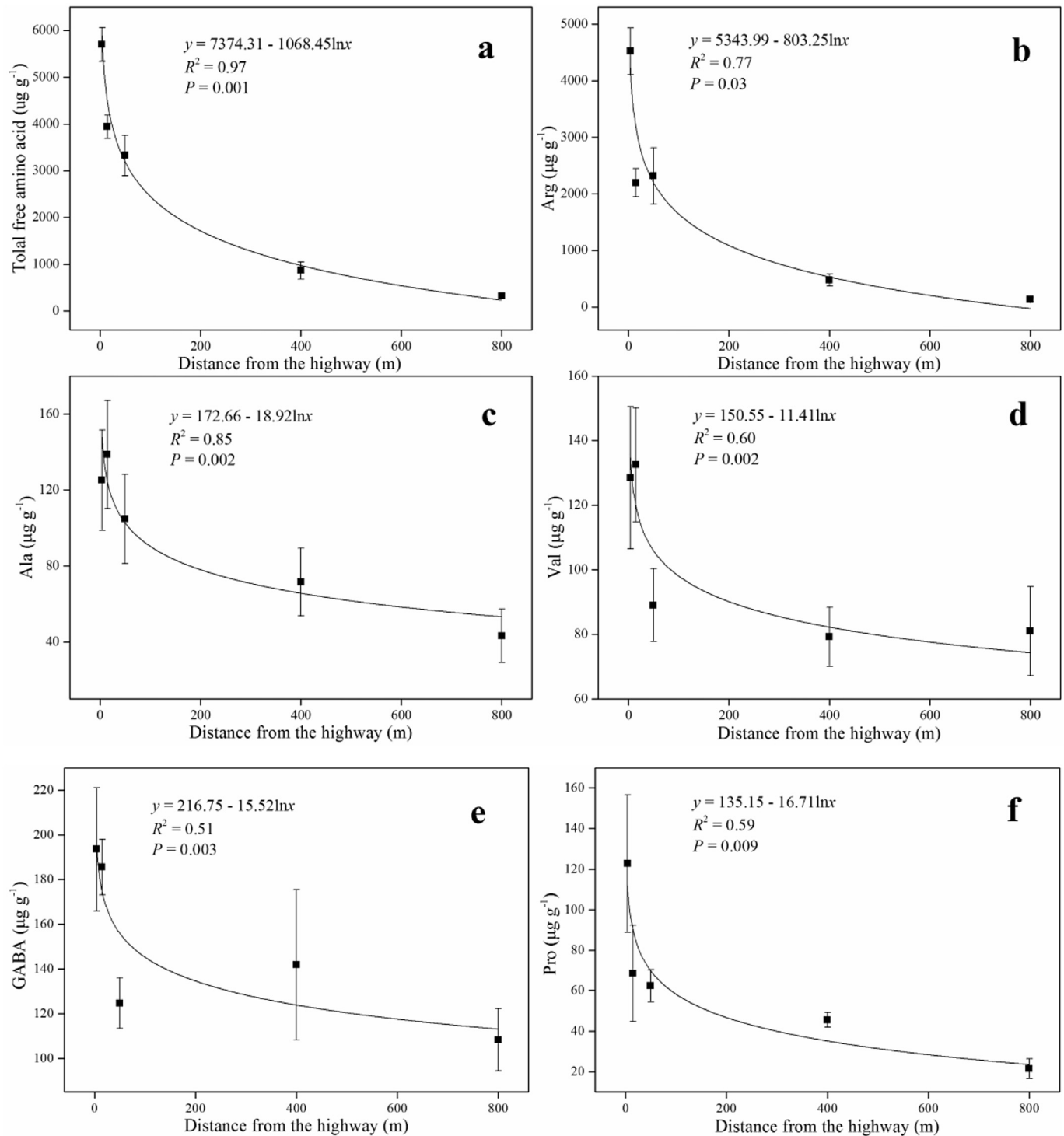


Fig. 3. Relationship between free amino acid concentrations in middle-aged needles and the distance from the highway for a) total free amino acid, b) Arg, c) Ala, d) Val, e) GABA and f) Pro. The vertical lines represent standard deviations.

further evidence for foliar free amino acid accumulation as an indicator of increased N deposition. However, free amino acid concentrations in new and old needles did not exhibit the similar correlation with distance from the highway as did free amino acid concentrations in middle-aged needles, suggesting that it may be improper to indicate N deposition using free amino acid concentrations in new and old needles. This might be attributable to the rapid use of amino acids in new needles for protein synthesis and

the export of amino acids from old needles to meet the N requirement of other tissues.

The N isotope technique has been used in a variety of terrestrial and aquatic ecosystems (Garten, 1993; Handley and Scrimgeour, 1997; Emmett et al., 1998). A prime example is the recognition of moss $\delta^{15}\text{N}$ values as a reliable and sensitive tool for identifying the sources of atmospheric N deposition because mosses mainly obtain their N requirements from atmospheric N deposition (Liu et al.,

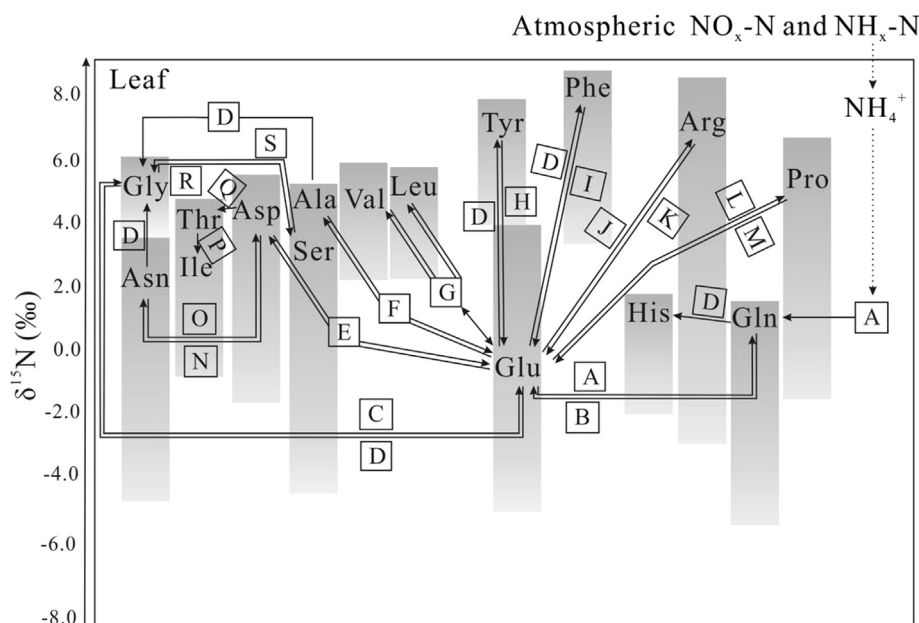


Fig. 4. Relationship between determined needle free amino acid $\delta^{15}\text{N}$ values (averaged all type of needles in each site) and known pathways of amino acid metabolism in plants across a distance gradient from the sites near the highway to the more distant sites. Variation in amino acid $\delta^{15}\text{N}$ values with increasing distance from the highway is indicated by grey bars (the lighter gradient colors represent the lower $\delta^{15}\text{N}$ values). Some key enzymes are shown in boxes: A, glutamine synthetase; B, glutamate synthase; C, glycine decarboxylase; D, transaminases; E, aspartate transaminase; F, alanine transaminase; G, branched-chain aminotransferase; H, tyrosine transaminase; I, phenylalanine: ammonia lyase; J, acetyl CoA-glutamate transacetylase and arginosuccinate lyase; K, arginase; L, Δ^1 -pyrroline-5-carboxylate synthetase and Δ^1 -pyrroline-5-carboxylate reductase; M, proline dehydrogenase and Δ^1 -pyrroline-5-carboxylate dehydrogenase; N, asparagine synthetase; O, asparaginase; P, threonine deaminase; Q, aspartate kinase and threonine synthase; R, serine hydroxymethyltransferase; S, serine: glyoxylate aminotransferase.

2008; Boltersdorf et al., 2014). However, the $\delta^{15}\text{N}$ values of vascular plant tissues can be affected by a range of environmental and biochemical processes and factors, which include the source of plant N uptake, mycorrhizal associations, root depth, N translocation in the plant, mineral N dynamics and aridity (Heaton, 1987b; Handley and Scrimgeour, 1997; Högborg, 1997; Michelsen et al., 1998; Emmerton et al., 2001; Choi et al., 2002). Although these source and process fractionations in the soil-plant system may cause changes in the foliar $\delta^{15}\text{N}$ values, a link between the $\delta^{15}\text{N}$ values of vascular plant leaves and atmospheric N pollutants has been corroborated by many studies. For example, Masson pine needles collected in an urban center had an average $\delta^{15}\text{N}$ value of +3.6‰, which is significantly different from the $\delta^{15}\text{N}$ value of rural needles (average of -2.7‰) (Xu and Xiao, 2017). Saurer et al. (2004) also reported that needles growing near a highway reflected the $\delta^{15}\text{N}$ values of traffic-derived NO_x . Because considerable amounts of atmospheric N compounds can be directly absorbed through foliar stomata, the isotope effect associated with atmospheric N uptake by leaves may be relatively small compared with the processes of N assimilation and translocation in the soil-plant system. Furthermore, N isotopic fractionation during soil and plant processes could not lead to significant differences in the needle $\delta^{15}\text{N}$ values between sites because only healthy Masson pines of similar ages and sizes were selected to minimize the influence of varying canopy height and root depth between sampling trees, and both soil $\delta^{15}\text{N}$ values (with the exception of site S1) and soil N concentrations were somewhat similar in all sites. Therefore, in this study, the large range in the $\delta^{15}\text{N}$ values reported for the needles suggests that needle $\delta^{15}\text{N}$ values may be highly sensitive to different types of atmospheric N pollutants. We can thus conclude that the difference between the $\delta^{15}\text{N}$ values of the needles and the soil increased with increasing distance from the highway as a result of the influence of atmospheric N pollution. Additionally, the $\delta^{15}\text{N}$ values in mosses from each site of leaf sample collection showed a

clearly decreasing trend, with more positive moss $\delta^{15}\text{N}$ values near the highway and ^{15}N -depleted moss $\delta^{15}\text{N}$ values at the more distant sites (Fig. 1). This also corroborates that a gradient of additional N compounds in the atmosphere clearly does exist excepting a significant decrease in atmospheric NO_2 concentration with increasing distance from the highway. More information about variations in atmospheric N sources with distance gradient from the highway can be obtained from the N isotope compositions of the free amino acids in needles of different ages.

The $\delta^{15}\text{N}$ values of all detectable free amino acids in the needles were found to be significantly positive at the site closest to the highway (S1) and to increase with increasing needle age. This pattern is consistent with that of the needle $\delta^{15}\text{N}$ values and the total needle free amino acid $\delta^{15}\text{N}$ values at site S1. More positive $\delta^{15}\text{N}$ values are generally associated with NO_x from traffic and industry emissions. For instance, averages of $+5.7 \pm 2.8\%$ and $+3.1 \pm 5.4\%$ were reported for the $\delta^{15}\text{N}$ values of traffic-derived NO_2 and NO , respectively (Ammann et al., 1999). The $\delta^{15}\text{N}$ values of traffic-derived NO_2 reported by Saurer et al. (2004) were in the range of +1.3‰ to +6.4‰, and isotopic compositions of NO_x sourced from coal combustion were +6‰ to +13‰ (Heaton, 1990). Numerous previous studies also suggested that greater uptake of atmospheric NO_x could significantly increase the leaf $\delta^{15}\text{N}$ values (Pearson et al., 2000; Xiao et al., 2011). Thus, the variations in the needle $\delta^{15}\text{N}$ values and free amino acid $\delta^{15}\text{N}$ values could be related to traffic-derived NO_x . After N compounds from atmospheric deposition are metabolized in leaves, the N can then be rapidly incorporated into amino acids (Baxter et al., 1992; Ballmoos et al., 1993). However, it is important to note that the process of amino acid metabolism can cause a large isotope fractionation. For instance, Gauthier et al. (2013) reported that the isotope fractionation between nitrate and Glu was 15.8‰, and the isotope fractionation associated with Asn synthesis from Asp was 36‰. The isotope effects between Glu and Asp, Pro, Phe, Leu, Gly and Ser in

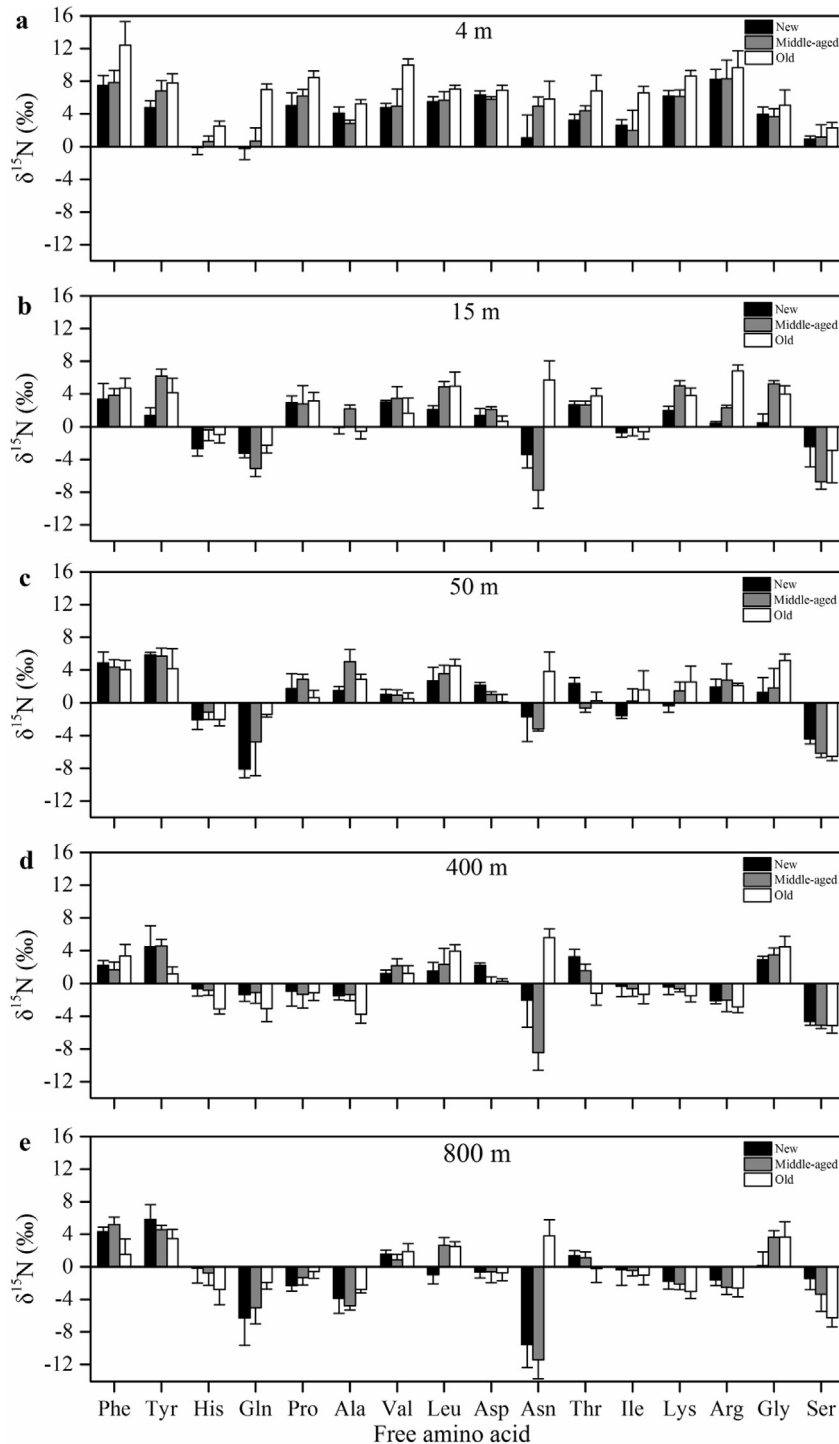


Fig. 5. The patterns of free amino acid $\delta^{15}\text{N}$ values for new, middle-aged and old needles from five sites at different distance from the highway: a) 4 m, b) 15 m, c) 50 m, d) 400 m and e) 800 m. The vertical lines represent standard deviations.

plant tissues were in the range of 2‰–8‰ (Styring et al., 2014a). But even so, fumigation experiments with $^{15}\text{NO}_2$ have demonstrated increases in the free amino acid $\delta^{15}\text{N}$ values in sunflower leaves (e.g., Gln, Pro, Ala, Ser, Gly, Glu and Asp) (Segschneider et al., 1995) and Norway spruce needles (e.g., Gln, Leu, Ile, Ala, Ser, Gly, Glu and Asp) (Nussbaum et al., 1993), which further indicates that there is an observable effect of atmospheric NO_x on foliar free amino acid $\delta^{15}\text{N}$ values. According to the above interpretation, we

could infer that when a greater portion of the foliar N requirement is obtained from the direct uptake of isotopically heavy $\text{NO}_x\text{-N}$ from atmospheric deposition than from soil N, any free amino acid ^{15}N depletion caused by process fractionation (during amino acid metabolism) or ^{15}N -depleted sources in needles would be balanced by the ^{15}N -enriched NO_x from traffic emissions. Additionally, since the leaves can absorb substantial quantities of N compounds via atmospheric deposition, foliar $\delta^{15}\text{N}$ values should be increasingly

influenced by the $\delta^{15}\text{N}$ value of deposited N from the atmosphere with longer exposure times (Gebauer et al., 1994). This suggests that when the N gained by needles from atmospheric deposition is ^{15}N -enriched, the needle $\delta^{15}\text{N}$ values and free amino acid $\delta^{15}\text{N}$ values should increase with increasing needle age. Therefore, the higher $\delta^{15}\text{N}$ values of foliar free amino acids and age-related free amino acid $\delta^{15}\text{N}$ values were typical of the influence of traffic-derived NO_x . Further support for this result was provided by the $\delta^{15}\text{N}$ values of the mosses collected around the selected trees. As shown in Fig. 1, more positive moss $\delta^{15}\text{N}$ values at site S1 clearly demonstrated a higher contribution of oxidized N species from traffic emissions to N deposition. Interestingly, although the slightly increased needle $\delta^{15}\text{N}$ values and needle total free amino acid $\delta^{15}\text{N}$ values with increasing needle age were observed at site S2, the age-related trend in the $\delta^{15}\text{N}$ values of most of the detectable free amino acids in the needles was not similar to that for the needles from site S1. This result suggested that site S2 was relatively less affected by NO_x from traffic emissions compared to the site near the highway.

All detectable free amino acid $\delta^{15}\text{N}$ values were significantly lower in needles collected from sites beyond 400 m from the highway than in needles collected close to the highway, and the $\delta^{15}\text{N}$ values of most of the free amino acids were depleted. Since atmospheric transport of pollutants would be strongly obstructed by the forest canopy (Saurer et al., 2004), the impact of NO_x from traffic emissions on needle $\delta^{15}\text{N}$ values and needle free amino acid $\delta^{15}\text{N}$ values was only evident in close proximity to the highway, and this significant influence of NO_x already disappeared in 400 m distance. This can be also demonstrated by the lack of a significant difference in moss $\delta^{15}\text{N}$ values (negative value) between sites S4 and S5 (Fig. 1). Reduced N species are known to commonly have negative $\delta^{15}\text{N}$ values (Liu et al., 2008). The $\delta^{15}\text{N}$ values of $\text{NH}_x\text{-N}$ from soil emissions and animal excrement are -5.8% to -3.3% and -15% to -4% , respectively (Freyer, 1978; Heaton, 1987a). Xiao and Liu (2002) reported an average of $-12.2 \pm 6.7\%$ for the $\delta^{15}\text{N}$ values of NH_4^+ in Guiyang rainwater. Therefore, the noticeably decreased and negative $\delta^{15}\text{N}$ values for most of the free amino acids in the sites beyond 400 m from the highway might reflect the variation in atmospheric N sources.

If needles are exposed to ^{15}N -depleted atmospheric N, a decrease in needle $\delta^{15}\text{N}$ values with increasing leaf age (longer accumulation time of atmospheric N for older leaves) would be expected. A similar age-dependent trend in the needle $\delta^{15}\text{N}$ values was also found by Gebauer and Schulze (1991) and Gebauer et al. (1994). They attributed the decrease in the $\delta^{15}\text{N}$ values from new to old needles to foliar uptake of isotopically lighter atmospheric N compared to N obtained from the soil. The $\delta^{15}\text{N}$ values of free His, Gln, Pro, Ala, Asp, Ile, Lys, Arg and Ser were negative in site S5 and a slightly decreasing trend in the needle $\delta^{15}\text{N}$ values and the needle free His, Asp, Ile, Lys, Arg and Ser $\delta^{15}\text{N}$ values from new to old leaves was also observed in this site, which may thus be attributable to greater uptake from ^{15}N -depleted atmospheric $\text{NH}_x\text{-N}$ by needles. Similarly, in a study carried out by Hofmann et al. (2003), a marked ^{15}N -depletion of free Asp, Gln, Pro, Ala, Asp, Ile, Lys, Pro, Val and Ser in moss tissues was found in an agricultural area (Menz, Mecklenburg, Germany) where reduced N species dominated. Although the reverse pattern in the needle and the total free amino acid $\delta^{15}\text{N}$ values was found in sites beyond 50 m from the highway, the total free amino acid $\delta^{15}\text{N}$ values may contribute small to the variation of the needle $\delta^{15}\text{N}$ values because only a quite small percentage of the total leaf N is contained in free amino acid N (less than 5% in this study) (Näsholm and Ericsson, 1990). Moreover, the above conclusion can also be confirmed by the similarity of the more negative moss $\delta^{15}\text{N}$ values at these sites to the $\delta^{15}\text{N}$ values of $\text{NH}_x\text{-N}$ (-5.8% to -3.3%) from soil emissions (Freyer, 1978) and by the fact

that there is no other source of $\text{NH}_x\text{-N}$.

It was unexpected that only a portion of the detectable free amino acid $\delta^{15}\text{N}$ values in the needles showed a consistent age-related $\delta^{15}\text{N}$ trend in sites beyond 50 m from the highway in spite of an evident decrease in the needle $\delta^{15}\text{N}$ values with increasing leaf age; moreover, there is a reverse pattern in the needle and the needle total free amino acid $\delta^{15}\text{N}$ values (the total free amino acid $\delta^{15}\text{N}$ values in middle-aged and old needles were higher than the corresponding needle $\delta^{15}\text{N}$ values). These discrepancy may have been caused by the $\delta^{15}\text{N}$ value of the N source and any isotope fractionation associated with the biosynthetic and metabolic pathways of amino acids. The most significant difference between the free amino acid $\delta^{15}\text{N}$ values of the new and old needles was found for Asn. N remobilized (e.g., Asn) from old leaves is a main contributor to the N requirements of other tissues, and the process of deamidation of Asn discriminates against ^{15}N , leaving the remaining Asn enriched in ^{15}N (Styring et al., 2014a). This can explain the prominent ^{15}N enrichment of Asn in old needles compared to in new needles. The deamination of Phe is catalyzed by phenylalanine ammonia-lyase to cinnamate, and the deamination of Tyr is catalyzed by tyrosine ammonia-lyase to 4-hydroxycinnamate. The kinetic isotope effect associated with these reactions can lead to marked depletion of ^{15}N in the product (Hermes et al., 1985; Styring et al., 2014b), resulting in a corresponding ^{15}N enrichment of the residual Phe and Tyr. Thus, the $\delta^{15}\text{N}$ values of aromatic amino acids Phe and Tyr are more positive than the $\delta^{15}\text{N}$ values of the other free amino acids. Similarly higher Phe and Tyr $\delta^{15}\text{N}$ values compared to most of the other amino acids were found by Smallwood et al. (2003) in tall *Red mangrove* leaves, and by Bol et al. (2002) in leaves of two grassland plants, *Juncus effusus* and *Lolium perenne*. Both Val and Leu are synthesized from pyruvate and receive their amino-N from the transamination of Glu, and therefore, factors effecting the isotope composition of pyruvate and transamination will contribute directly to the $\delta^{15}\text{N}$ values of Val and Leu (Binder et al., 2007; Lynch et al., 2016). These processes potentially caused a relative ^{15}N enrichment of Val and Leu. Gly and Ser involve the photorespiratory cycle in the leaves. The $\delta^{15}\text{N}$ value of Gly is positive and always higher than that of Ser (^{15}N -depleted except for in site S1). This is possibly explained by either (1) the isotope effect associated with transamination from Glu to produce Gly (Tcherkez, 2011), or (2) discrimination against ^{15}N associated with the reaction that converts Gly to Ser (Styring et al., 2014a). Clearly, N isotope fractionation associated with the biosynthetic and metabolic pathway of amino acids in needles can cause differences in the $\delta^{15}\text{N}$ values between foliar free amino acids, and the ^{15}N enrichment of Asn, Phe, Tyr, Val, Leu and Gly caused by these processes cannot be balanced by ^{15}N -depleted $\text{NH}_x\text{-N}$ source in the atmosphere. In spite of this, the fact that the evident ^{15}N depletion in foliar total free amino acids and free His, Gln, Pro, Ala, Asp, Ile, Lys, Arg and Ser in sites beyond 400 m from the highway unambiguously indicates that NH_3 from soil emissions contribute significantly to N deposition at these sites.

5. Conclusion

The pattern of markedly decreased free amino acid concentrations in needles with increasing distance from the highway indicated a variation in atmospheric N deposition. We also found that a larger decreasing proportion occurred in foliar free amino acid concentrations (especially for Arg) rather than in foliar total N concentrations in response to decreased atmospheric N inputs. This suggests that the concentrations of free amino acids in leaves can be used as more sensitive indicators of atmospheric N pollution compared with foliar total N concentrations. In addition, free amino acid concentrations (e.g., total free amino acid, Arg, GABA, Val, Ala

and Pro) in new and old needles did not show a significant correlation to distance from the highway as did free amino acid concentrations in the middle-aged needles, suggesting that free amino acid concentrations in middle-aged needles may be preferable for indicating atmospheric N deposition. This is likely due to the rapid use of amino acids in new needles for protein synthesis and the export of amino acids from old needles to meet the N requirement of other tissues.

The present study is the first to investigate the isotope compositions of free amino acids in needles of different ages. It has been shown that there were significant differences in the $\delta^{15}\text{N}$ values of needle free amino acids with increasing distance from the highway. The $\delta^{15}\text{N}$ values of all detectable free amino acids in the needles collected at the site closest to the highway were significantly more positive than those collected at the more distant sites and increased with increasing needle age. This indicated that their isotope ratios in this site were much more affected by isotopically heavy NO_x from traffic emissions than the other sites. In sites beyond 400 m from the highway, not all of the detectable free amino acid $\delta^{15}\text{N}$ values of the needles exhibited an identical age-related $\delta^{15}\text{N}$ trend, while the evident ^{15}N depletion in needle total free amino acid, His, Gln, Pro, Ala, Asp, Ile, Lys, Arg and Ser clearly indicated that the $\delta^{15}\text{N}$ values of needle free amino acids were affected to a greater extent by ^{15}N -depleted atmospheric $\text{NH}_x\text{-N}$. The above results can be also confirmed by the pattern of the $\delta^{15}\text{N}$ values in mosses from each site of leaf sample collection. Therefore, this study presents novel evidence that the $\delta^{15}\text{N}$ values of free amino acids in needles are able to identify atmospheric N sources. Further study of this topic could be in the evaluation of variation in leaf metabolic fluxes under condition of high N deposition, so that plant adaptations to stress of high N deposition would be expected to be reflected. This may also hold considerable promise to indicate the effects of atmospheric N deposition on plant physiology and N cycles.

Acknowledgments

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