Free amino acid concentrations and nitrogen isotope signatures in *Pinus massoniana* (Lamb.) needles of different ages for indicating atmospheric nitrogen deposition☆

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ABSTRACT

Free amino acid concentrations and nitrogen (N) isotopic composition in new current-year (new), mature current-year (middle-aged) and previous-year (old) Masson pine (*Pinus massoniana* Lamb.) needles were determined to indicate atmospheric N deposition in Guiyang (SW China). In different areas, free amino acids (especially arginine) concentrations in new and middle-aged needles were higher than in old needles, and the variation of free amino acids (especially arginine) concentrations in new and middle-aged needles was also greater than in old needles. This indicate that free amino acids in new and middle-aged needles may be more sensitive to N deposition compared to old needles. Moreover, concentrations of total free amino acids, arginine, histidine, γ-aminobutyric acid and alanine in middle-aged needles exhibited a strong relationship with N deposition ($P < 0.05$). Needle $\delta^{15}N$ values showed a strong gradient from central Guiyang to the rural area, with more positive $\delta^{15}N$ (especially in old needles) in the city center (0–5 km) and more negative $\delta^{15}N$ (especially in old needles) in rural area (30–35 km). These suggest that N deposition in the urban center may be dominated by ^{15}N-enriched NO_x-N from traffic exhausts, while it is dominated by isotopically light atmospheric NH_x-N from agriculture in rural area. Soil $\delta^{15}N$ decreased slightly with distance from the city center, and the difference in $\delta^{15}N$ values between the soil and needles (especially for old needles) increased significantly with the distance gradient, indicating that atmospheric N deposition may be an important N source for needles. This study provides novel evidence that free amino acids in needles and age-dependent needle $\delta^{15}N$ values are useful indicators of atmospheric N deposition.

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1. Introduction

By 2030, worldwide atmospheric N deposition may increase 1.5- to 2-fold over 2000 levels due to high N emission from urbanization and agricultural activities (Reay et al., 2008). Dramatically increased N deposition is result of poor air quality in most cities and represents a threat to the health of natural ecosystems (Maskell et al., 2010; Liu et al., 2013). For instance, it is known that enhanced N deposition can increase N concentrations in many types of leaves (Leith et al., 1999; Hicks et al., 2000; Liu et al., 2013). Plants can absorb atmospheric inorganic N (e.g., NO_x and NH_3) through leaf uptake, and these N compounds are rapidly transformed into amino acids (Geßler et al., 2002; Yoneyama et al., 2003). Furthermore, Lockwood et al. (2008) found that leaves can absorb atmospheric organic N compounds produced by the atmospheric oxidation of volatile organic compounds and convert them into useful leaf amino acids (e.g., glutamate and aspartate). More importantly, atmospheric organic N compounds account for twenty per cent of atmospheric nitrogen oxides ($NO_y = PANs + RO_2NO_2 + RONO_2 + HNO_3 + HONO + N_2O_5 + NO_2 + NO + \dots$) and may be an important N source for plants (Day et al., 2003; Murphy et al., 2006). Because plants can obtain a certain proportion of required N from direct atmospheric N uptake by leaves, elevated free amino acid concentrations in plant leaves have been correlated with higher atmospheric N deposition (Calanni et al., 1999; Power and Collins, 2010). Several studies have suggested that free amino

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acid concentrations in leaves provide good indications of increased N deposition (Pietilä et al., 1991; Pitcairn et al., 2003).

Changes in leaf tissue N concentrations (especially in lichens and bryophytes) have frequently been used as bio-monitors in atmospheric N pollution studies (Schaberg et al., 1997; Gombert et al., 2003; Xiao et al., 2010a). A surplus N supply can also cause significant changes in the relative concentrations of leaf free amino acids, as these amino acids can serve as a storage form of N for protein synthesis and future growth (Pietilä et al., 1991; Nordin et al., 2001). More interestingly, foliar free amino acid concentrations had much higher increasing proportion than total leaf N concentrations in response to N inputs. For example, a study by Nordin et al. (1998) found that simulated N deposition of 50 kg N ha^{-1} yr^{-1} caused increases of over 50% in the free amino acid concentrations of *Deschampsia flexuosa* leaves. By comparison, increases in foliar N concentrations were less than half this amount. Therefore, the use of foliar free amino acids in vascular plants as bio-monitors of atmospheric N pollution should be promoted especially in heavily polluted areas where lichens and bryophytes will not grow.

As a rule, the $\delta^{15}N$ values associated with NO_x-N from fossil fuel combustion are relatively positive (Heaton, 1990), while those associated with agricultural NH_x-N from animal excrement and fertilizer use are relatively depleted (Freyer, 1978; Heaton, 1987). These isotopic data provided reliable integrated information on the potential N sources of atmospheric inputs in several terrestrial environments. Bryophytes obtain N mainly from the atmosphere and have very low isotopic discrimination during N uptake (Bragazza et al., 2005). Therefore, many previous studies have shown that moss $\delta^{15}N$ provides reliable information to distinguish sources of N deposition (Dawson et al., 2002; Solga et al., 2005). In contrast, $\delta^{15}N$ values in the leaves of soil-grown plants are the integrated results of plant N uptake, root depth, plant mycorrhizal status, isotopic fractionation in N uptake and even the influence of canopy trees (Högberg, 1997; Asada et al., 2005; Xiao et al., 2011). Therefore, previous studies on $\delta^{15}N$ in vascular plant leaves for indicating atmospheric N deposition are very limited. Although there is some evidence that the $\delta^{15}N$ values in vascular plant leaves can reflect the sources of atmospheric N pollution (especially for plants that can obtain much N from the atmosphere by direct leaf uptake), these studies were mainly concentrated on forest ecosystems or point source pollution. For example, Xiao et al. (2011) found that more positive $\delta^{15}N$ values in camphor leaves occurred in the vicinity of the road, while camphor leaves sampled near a chemical fertilizer plant had strongly negative $\delta^{15}N$ values. The $\delta^{15}N$ values in *Picea abies* needles (sampled in a mixed forest) increased from −4.4‰ to +1.3‰ with increasing distance from the highway in a study by Saurer et al. (2004).

Free amino acid concentrations and N isotopic compositions in moss tissue are potentially useful tools for providing some specific information about environmental and ecological processes (Pitcairn et al., 2003; Zechmeister et al., 2008; Boltersdorf et al., 2014). Vascular plants are less commonly used to indicate atmospheric N pollution, and much less work has been conducted to investigate the foliar amino acid concentrations and $\delta^{15}N$ of vascular plants in urban environments that contribute significantly to local biodiversity. In this paper, *Pinus massoniana* (Lamb.), which is widely distributed in Guiyang city, was selected for leaf sampling. Of particular interest are: (1) whether free amino acids in needles can be used as indicators of N deposition in areas susceptible to the heavy atmospheric reactive N input; (2) how the needle $\delta^{15}N$ changes with needle age and what factors affect it; and (3) whether needle $\delta^{15}N$ values can be used to identify atmospheric N sources?

2. Materials and methods

2.1. Sampling area description

Guiyang city, the provincial capital of Guizhou (SW China), is located in a Karst region with an average altitude of 1250 m. This city is characterized by a typical subtropical monsoon climate with an annual mean temperature of 15.3 °C and an annual mean relative humidity of 86%. In the Guiyang area, annual precipitation is 1175 mm, and the prevailing wind direction is the southeast in the summer and the northeast all year (Guiyang Environmental Protection Bureau, 2006). The study area lies between 26°19′ N - 26°45′ N latitude, and between 106°19′ E − 106°47′ E longitude (Fig. 1). Yellow soil (in the Chinese classification system) is the major soil type in this study area. The soil is acidic and strongly weathered, with low base saturation and high aluminium concentration (Larssen et al., 1998). Guiyang is densely populated and has high-density traffic. The urban center (the northern part of Nanming and the southern part of Yunyan) have population density of 30,000/km^2 and more than 100,000 motor vehicles (Li et al., 2012). The population of motor vehicles was 225,400 in 2005, increased to 610,800 in 2010 (He, 2013), and exceeded 790,000 in 2014 (data from http://www.gzgov.gov.cn/xwzx/gszdt/gy/201506/t20150626_182223.html). Of these, a considerable number of vehicles fail to meet the motor vehicle exhaust gas standard. The average concentration of NO_2 in the atmosphere of urban area increased from 19 μg m^{-3} in 2006 to 35 μg m^{-3} in 2010 because of high-density traffic (Guiyang Environmental Protection Bureau, 2007; Tian et al., 2013). In the last 10−15 years, the rate of wastewater treatment was comparatively low (17% in 2004 and 20% in 2005), and higher NH_x-N deposition in the urban area of Guiyang was largely due to discharge of domestic wastewater with high NH_3 concentrations. However, in recent years, wastewater has been routed to centralized treatment, and the rate of wastewater treatment has increased to 90% (Guiyang Environmental Protection Bureau, 2016). According to data from He (2013) and Tian et al. (2013), total emissions of NO_x in Guiyang in 2010 were 36.6 kt yr^{-1}, while NO_x from vehicle emission was 20.2 kt yr^{-1}. In addition, emissions of NO_x were 12-fold higher than NH_3 emissions in 2010 throughout the province (MEP, 2011).

2.2. Sample collection and treatment

The sampling season was May 2015. None of the selected sampling sites was disturbed by local point or non-point sources. All Masson pine trees selected for leaf sampling were approximately 20 years old and approximately 13 m in height. Approximately 4−8 g of new current-year needles (new, verdant needles from the shoot apex that were shorter than the needles below them), mature current-year needles (middle-aged, verdant needles found below the new needles) and previous-year needles (old, non-verdant needles in the lower layers, which were grown in 2014) were collected respectively from outer branches (approximately 12 m above the ground) at south, west, east, and north directions. We collected 5−10 representative samples for each type of leaf from 4 to 6 Masson pine trees at each sampling area. New current-year needles and old needles were not available from the sampled trees in the 11−18 km and 24−30 km zones. Moss samples (*Haplocladium microphyllum* (Hedw.) Broth) were collected immediately from the surrounding exposed rocks after the leaves were sampled at each site. The distance between mosses and trees chosen for leaf sampling was less than 50 m at most of sampling sites. 3−5 moss samples were collected at each area (3−5 subsamples were combined into one sample). Urban samples were mainly collected from
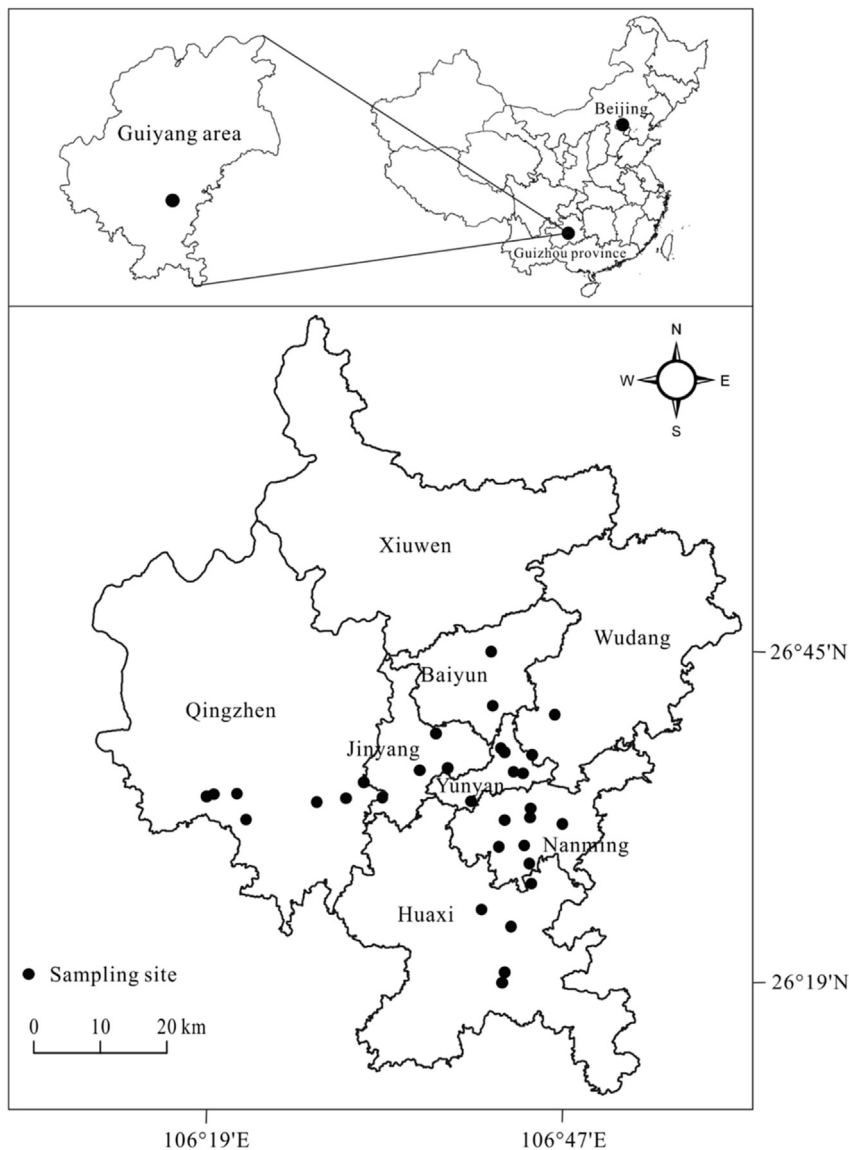
Fig. 1. Map showing the location of Guiyang area and leaf sampling sites.

parks or hills. All sampling sites were located at least 100 m from main roads. Only healthy samples were taken, and all samples were taken under stable weather conditions without rain. Soil (approximately 100 g) was sampled from the rooting zone (at depths of 0—10 cm) after sampling the needles at each site. Roots and leaf litter were removed from soil samples immediately.

Fresh samples were placed in clean plastic bags and stored in a chilled box immediately. In the lab, all samples were washed with deionized water. Each washed sample was divided into two halves; half of each sample was dried at 80 °C for 24 h, and the other half was freeze-dried. Soil samples were also dried at 80 °C for 24 h. Then all samples were ground to a fine homogeneous powder. Freeze-dried samples were frozen immediately in liquid N and stored at −80 °C until further use.

2.3. Analysis of N and $\delta^{15}N$

N concentration (%; dry weight) analysis was carried out using a vario MACRO cube elemental analyzer (Elementar, Frankfurt, Germany) with analytical precision of 0.02%. The $\delta^{15}N$ values of samples were analyzed using a Thermo MAT 253 mass spectrometer (Thermo Finnigan, Bremen, Germany) coupled to a Flash EA 2000 elemental analyzer (Thermo Scientific, Bremen, Germany). Calibration of $\delta^{15}N$ values with ammonium sulfate (IAEA-N-1, $\delta^{15}N = +0.4‰$) and potassium nitrate (IAEA−NO−3, $\delta^{15}N = +4.7‰$) standards was performed. The analytical precision (SD, n = 3) of $\delta^{15}N$ was better than $\pm0.1‰$. N concentrations and $\delta^{15}N$ values were reported as averages of 3—5 replicated measurements for each sample.

2.4. Estimation of N deposition

Atmospheric N deposition (N_{dep}) at each sampling area was estimated based on moss tissue N concentrations (N_m; %) from this study and positive and significant linear correlation between atmospheric N deposition (wet and dry) values from some sites of China compiled by Xiao et al. (2010a) and the corresponding moss N concentrations: $N_m = 0.052N_{dep} + 0.73$ ($R^2 = 0.70$, $P < 0.001$).

The N deposition values calculated are shown in Table 1.

Table 1
Estimated and published atmospheric N deposition in different sampling areas.

Sites (km)	Estimation of atmospheric N deposition (kg N ha^{-1} yr^{-1})	Reported in the literature (kg N ha^{-1} yr^{-1})	References
0–5	32.22 ± 3.11	29.21 ± 6.17	Liu et al. (2009)
5–11	21.63 ± 2.86	21.98 ± 8.30	Liu et al. (2009)
11–18	17.84 ± 0.59	17.93 ± 7.49	Liu et al. (2009)
18–24	11.68 ± 2.26	11.95 ± 3.95	Liu et al. (2009)
24–30	19.01 ± 0.83	16.70 ± 2.76	Liu et al. (2009)
30–35	22.90 ± 4.56	20.86 ± 3.72	Liu et al. (2009)

2.5. Reagents and standards for amino acid analysis

All amino acid standards (≥99% purity) were from Sigma-Aldrich. Methanol, acetonitrile and Na_2PO_4 were HPLC-grade reagents from Sigma-Aldrich. Trifluoroacetic acid (TFA) (99% purity) was from Sigma-Aldrich. Phosphate buffer was prepared by mixing 10 mol L^{-1} NaOH solution in Na_2PO_4 solution to 0.04 mol L^{-1} and adjusting the pH to 7.8. Borate buffer (0.4 mol L^{-1}, pH 10.2), *o*-phthalaldehyde (OPA) reagent (10 mg mL^{-1}) in 0.4 mol L^{-1} borate buffer and 3-mercaptopropionic acid (3-MPA), and 9-fluorenylmethyl chloroformate (FMOC) (2.5 mg mL^{-1}) in acetonitrile were from Agilent (Agilent Technologies, Palo Alto, USA).

2.6. Analysis of amino acid

A 0.15 g powdered sample (200 μL, 1 nmol μL^{-1} α-aminobutyric acid and sarcosine added as internal standards) was shaken with the extractant (1.8 mL, 10% v/v TFA) in 5 mL tubes in a reciprocating shaker for 5 min. The homogenate was centrifuged at 12,000 rpm for 15 min (4 °C) after ultrasonication for 25 min (4 °C), and the supernatant solution was collected. The remaining sample was re-extracted using 2 mL TFA (10% v/v) in the same manner, and the supernatants were combined. All supernatants were filtered through a 0.22 μm membrane, and the collected solution was purified using 4 mL Amicon Ultra 3K centrifugal filter tubes (Millipore, Billerica, USA). Purified solutions were analyzed by HPLC directly.

The amino acids were analyzed using an HPLC system (Agilent Technologies, Palo Alto, USA). The equipment consisted of a G1311B quatpump, a G1316A column oven, a G1329B autosampler and a G1321B FLD. The amino compounds detected by this method include 28 amino acids (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)), and the detection limit of the HPLC is 4.5 μmol L^{-1}.

The analysis of amino acid extracts (1 mL) was performed according to the developed Agilent amino acid HPLC method. The separation of amino acids was completed on a Zorbax Eclipse AAA-C18 guard column (4.6 × 12.5 mm, 5 μm, Agilent) and a Zorbax Eclipse AAA-C18 column (4.6 × 150 mm, 3.5 μm, Agilent). Amino acid analysis was achieved with automation and online derivatization using OPA and FMOC. Specific derivatization and injection methods were as follows: 0.5 μL sample, 2.5 μL borate buffer (0.4 mol L^{-1}, pH 10.2), 0.5 μL OPA, 0.5 μL FMOC and 32 μL ultrapure water were mixed. The reaction mixture (18 μL) was immediately injected into the HPLC system. The mobile phase A was composed of methanol/acetonitrile/water (45/45/10, v/v/v), and mobile phase B was phosphate buffer (0.04 mol L^{-1}, pH 7.8). The temperature of the column oven was set to 40 °C. The fluorescence wavelength 450 nm was programmed to switch to 305 nm at exactly 14.9 min.

The elution linear gradient was: 0–1.9 min, solvent A 0%; 1.9–18.1 min, solvent A increased to 57%; 18.1–18.6 min, solvent A increased to 100%; 18.6–22.3 min solvent A maintained at 100%; 22.3–23.2 min, solvent B increased from 0% to 100%; 23.2–26 min, solvent B maintained at 100%. From 0 to 26 min, flow maintained at 2 mL min^{-1}. We found that the coefficients of determination (R^2) were 0.9993–0.9999 for the calibration curves of all standards. The average recovery of amino acids was 94%.

2.7. Data analysis

All statistical procedures were performed using SPSS version 19 (IBM, Chicago, USA), and graphs were created with Origin 9.0 (OriginLab Corporation, Massachusetts, USA). The data were processed using one-way ANOVA with a Tukey-HSD test to compare differences between groups of samples, and the results were accepted for *P* values < 0.05.

## 3. Results

3.1. Needle, moss and soil N

N concentrations in needles ranged from 1.2% to 1.7% (Fig. 2 a). In general, the highest mean N percentage in the needle was found in new needles, and decreased with increasing leaf age. Higher average N concentrations in middle-aged needles occurred in the urban center (0–5 km) and lower concentrations at area of 18–24 km (Fig. 2 a).

Moss N concentration varied from 1.34% to 2.68%. The highest mean moss N concentration (2.41± 0.16%) among all areas from central Guiyang to the rural area occurred in the urban center, and the average moss N concentrations showed a decreasing trend from the urban center to the rural area. However, the lowest mean value (1.34± 0.12%) did not occur in the rural area (30–35 km) but in the area of 18–24 km, indicating a spatial trend of atmospheric N deposition in Guiyang, as shown in Fig. 2 c.

Soil N concentrations were in the range of 0.09%–0.28%, and N concentrations in the soil between sampling areas were found no significant difference (*P* > 0.05) (Table 2).

3.2. Needle free amino acids

Twenty-two amino acids were detected in the needles using the method of amino acid extraction and analysis described in this study. Those amino acids included Asp, Gln, Asp, Phe, Glu, Leu, Ser, Arg, Try, Gly, Orn, Thr, Lys, Ala, Pro, Cit, Val, Ile, Tyr, His, Met and GABA. Orn, Met and Cit were only found in a few samples with relatively high foliar N concentrations. Concentrations of Ile, Lys, Tyr and Trp in some samples were below the detection limit of the HPLC. Fig. 4 shows concentrations of major free amino acids in needles. Arg (28.7–5549.2 μg g^{-1}), Asp (104.3–471.0 μg g^{-1}), Glu (182.7–557.6 μg g^{-1}), Gln (60.7–762.7 μg g^{-1}) and GABA (110.9–342.1 μg g^{-1}) were present in relatively high concentrations, and the above mentioned free amino acids accounted for
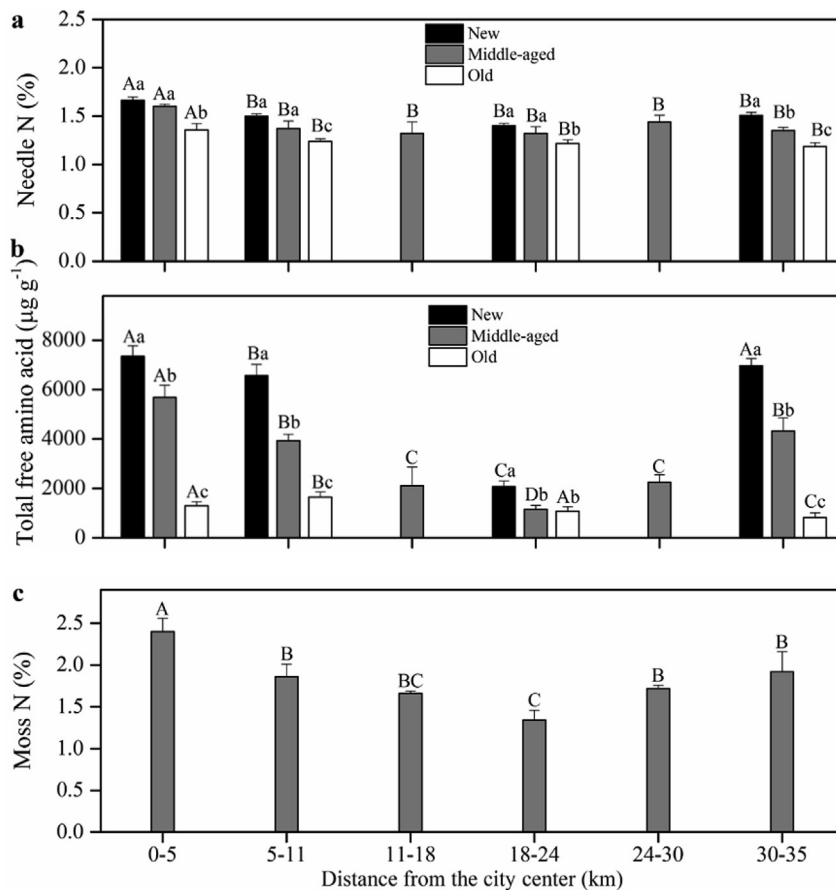
Fig. 2. Nitrogen (%; dry weight) (a) and total free amino acid (b) concentrations in different ages needles and N (%; dry weight) concentrations in mosses (c) from central Guiyang to rural area. Significant and spatial differences in the same age needles and mosses were marked with uppercase letters, while among the ages needles were marked with lowercase ones, ($P < 0.05$). The vertical lines represent standard deviations.

Table 2
Mean N concentrations and $\delta^{15}N$ (‰) in soils from central Guiyang to rural area (in parentheses minimum and maximum values).

Soil	0—5 km	5—11 km	11—18 km	18—24 km	24—30 km	30—35 km
N conc. (%; dry weight)	0.20 (0.19; 0.21)	0.18 (0.14; 0.21)	0.22 (0.14; 0.29)	0.19 (0.17; 0.21)	0.21 (0.18; 0.29)	0.17 (0.09; 0.22)
$\delta^{15}N$ (‰)	+5.6 (+4.6; +6.8)	+5.0 (+4.3; +6.7)	+3.9 (+1.4; +6.9)	+4.6 (+3.7; +6.1)	+3.8 (+2.2; +4.9)	+4.4 (+3.5; +4.9)

almost 90% of the total free amino acid concentrations in new and middle-aged needles. A strong accumulation of free amino acids (especially for Arg) was found in new and middle-aged needles, and the variations for other free amino acids were much less pronounced than for Arg (Fig. 4). The changes in N concentrations in needles were significantly smaller than the total free amino acid concentrations along the distance gradient (Fig. 2). In addition, some free amino acid concentrations (GABA, Arg, Val, Ala, His and total free amino acid) in needles presented a strong relationship with total N deposition ($P < 0.05$) (Fig. 3).

3.3. Needle and soil $\delta^{15}N$

The $\delta^{15}N$ values in needles ranged between −5.4‰ and +6.5‰, and needle $\delta^{15}N$ values showed a strong gradient, with more positive needle $\delta^{15}N$ (especially in old needles) in the urban center (0—5 km) and ^{15}N-depeleted needles (especially in old needles) in the rural area (30—35 km) (Fig. 5). Furthermore, the differences in $\delta^{15}N$ values between new and old needles at 0—5 km and 30—35 km areas were larger than those in the 5—11 km and 18—24 km areas.

By comparison, the average $\delta^{15}N$ value of soil samples only showed a slightly decreasing trend from urban to rural area, and the decrease was not significant ($P > 0.05$) (Table 2). Thus, the differences in soil and needle $\delta^{15}N$ values (especially for old needles) should increase significantly with increasing distance from the city center. Interestingly, the $\delta^{15}N$ values in needles were lower than that in soils across all sampling sites.

4. Discussion

Foliar N concentrations have been used to indicate early stages of N accumulation and detect the level of atmospheric N deposition. As shown in this study, N concentrations in middle-aged needles were related to estimated atmospheric N deposition ($P < 0.05$) (Fig. 3 a). Previous studies have also shown that highly consistent responses of leaves (including needles) to high N inputs (achieved by anthropogenic addition) was significantly higher foliar N and free amino acids concentrations (Näsholm et al., 1994; Edfast et al., 1996; Magill et al., 2000; Bauer et al., 2004). In fact, under natural conditions with low to moderate range of N inputs, extensive
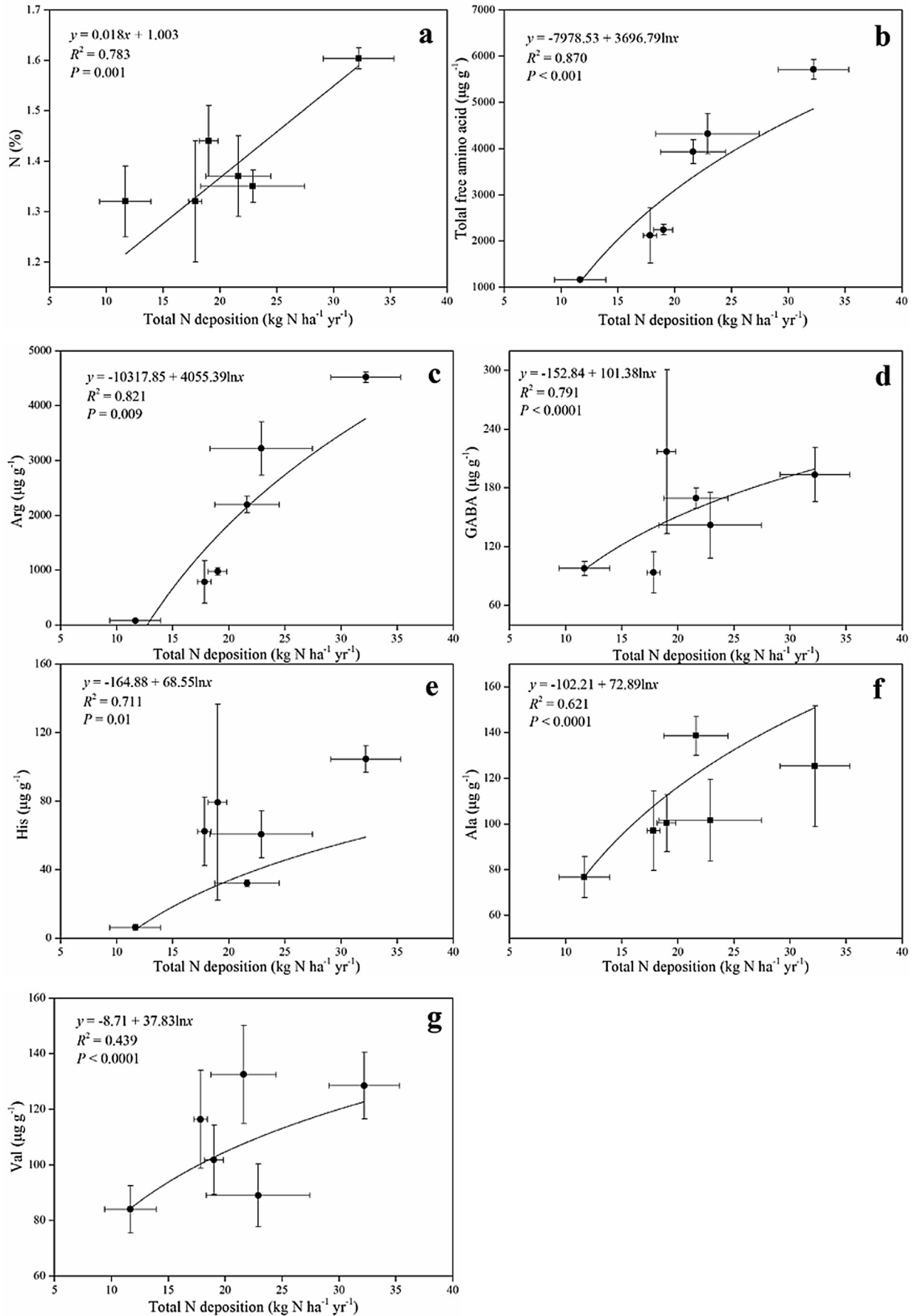
Fig. 3. Relationships between N and free amino acid concentrations in middle-aged needles and total N deposition for a) N, b) total free amino acid, c) Arg, d) GABA, e) His, f) Ala and g) Val. The vertical lines represent standard deviations.

studies have found that foliar N concentrations are relatively insensitive, instead significant changes in foliar free amino acids concentrations were observed (Huhn and Schulz, 1996; Nordin et al., 1998; Bauer et al., 2000; Aber et al., 2003). As in this investigation, the Arg concentration in the needles showed the most significant change from central Guiyang to the rural area, followed by Gln, Glu, Asp, GABA, Ala, Val and Pro. And the changes of free amino acid concentrations in needles were significantly larger than that of N concentrations. A plausible explanation is that Arg with favorable C/N ratio (1.5) is preferential N storage and transport compound, and other amino acids may also play a similar role (Huhn and Schulz, 1996). On the other hand, leaves can store N in the form of inorganic N (e.g., nitrate) and organic N (e.g., protein and amino acids) under relatively high N input. The use of inorganic N has clear trade off due to high maintenance costs, while organic N (especially amino acids) is much more favorable and is readily available for plant growth (Pate and Layzell, 1990). Moreover, a much smaller percentage of total tissue N is contained in free amino acids (Näsholm and Ericsson, 1990). Atmospheric N compounds (such as NH_3, NO, NO_2, HNO_3, RO_2NO_2 and $RONO_2$) are absorbed into the leaves by stomata and cuticle to the plant metabolism. A significant change in needle free amino acid concentrations will be observed when atmospheric N inputs change (e.g., in the different areas of this study). The above discussion may also imply that plants are usually able to adapt to a broad range of N inputs, but over a certain critical N load value, this adaptation may cause a significant increase in foliar N concentrations by changing N uptake and allocation. For example, earlier studies have reported that adaptation may involve higher N concentrations in *Sphagnum* tissue (Limpens and Berendse, 2003). In contrast, under natural conditions with low to moderate range of N inputs, foliar N concentration was relatively insensitive, and significant changes in foliar free amino acid concentrations would be observed, as mentioned above.

In this study, we also found that N concentrations (%) in needles slightly decreased with increasing leaf age from center city to rural areas and that free amino acid concentrations significantly decreased from new to old needles at all sampling areas, which may be attributed to the fact that N compounds derived from atmospheric deposition were not accumulated in old needles, but were transported to other tissues. The uptake of atmospheric N compounds through the needles is considerable, therefore, older needles had been accumulating atmospheric N compounds for longer time (Gebauer et al., 1994). The absorption and accumulation of N compounds from the atmosphere should be a joint process. If those N compounds were not transported, N and free amino acid concentrations in needles should increase continuously with increasing needle age (longer time of exposure). Thus, N and free amino acids concentrations in old needles should be higher than those in new needles, while this result was not observed. Amino acids and protein are much more readily available for plant growth compared to inorganic N (Pate and Layzell, 1990), therefore, foliar free amino acid concentrations decreased more dramatically than foliar N concentrations from new to old needles. The age differences in foliar N concentrations in this study are in agreement with other reports (Gebauer and Schulze, 1991; Kuang et al., 2011), but no studies have focused on changes in leaf free amino acid concentrations with leaf age.

In urban settings, heavy pollution and habitat availability have severely limited the use of bryophytes as bio-indicators of atmospheric N pollution. However, previous studies have found a link between free amino acid concentrations in vascular plant leaves (e.g., Norway spruce, Scots pine trees and heathland vegetation) and atmospheric N deposition (Edfast et al., 1990; Huhn and Schulz,

1996; Van den Berg et al., 2008). Power and Collins (2010) also reported significant relationships between free Phe ($R^2 = 0.774$), Glu ($R^2 = 0.661$) and Gly ($R^2 = 0.528$) concentrations in *Calluna* leaves and the level of atmospheric N deposition from central London to rural area. In this study, the estimated mean level of total atmospheric N deposition varied from 11.68 ± 2.26 kg N ha^{-1} yr^{-1} to 32.22 ± 3.11 kg N ha^{-1} yr^{-1}, which were similar to previous reports (Table 1). We found a strong relationship between free amino acid concentrations (total free amino acid, Arg, GABA, Ala, Val and His) in middle-aged needles and estimated total atmospheric N deposition (Fig. 3). Significant variations in concentrations of total free amino acid, free Arg, GABA, Ala, Val and His in middle-aged needles may be explained by atmospheric N deposition, suggesting that these free amino acids in needles may be useful indicators of atmospheric N deposition. Interestingly, plants can also respond to other environmental stresses (e.g., pest damage, pathogens, cold, heat, drought, salt and metal) with changes in amino acid composition, and a link between tissue amino acid composition and plant damage has been demonstrated (Sagisaka, 1974; Nordin et al., 1998; Di Martino et al., 2003; Sharma and Dietz, 2006; Xing et al., 2007). Therefore, it is possible that changes in the composition or size of needle free amino acid pool not only reveal level of atmospheric N deposition but also contribute physiological information about potential impacts of elevated N deposition to Masson pine.

Soil $\delta^{15}N$ was higher than needle $\delta^{15}N$ at all sites. This pattern seems to be a common phenomenon not only for coniferous trees, but also for other vegetation (Gebauer and Dietrich, 1993; Miller and Bowman, 2002). Ammann et al. (1999) reported that higher $\delta^{15}N$ values in soil compared to needles both near and far from highways, and a study by Xiao et al. (2011) also showed that soil $\delta^{15}N$ values were higher than camphor leaf $\delta^{15}N$ values near a road and a chemical fertilizer plant. It is well known that the organic layer can provide N input into the mineral soil and lead to ^{15}N enrichment of the lower soil layer (0–10 cm) (Ammann et al., 1999). Another possible reasons caused soil ^{15}N enrichment were discussed as follows. The major pathways of N loss and N metabolism in soil, e.g., ammonia volatilization, nitrification, microbial immobilization of soil ammonium and denitrification, could cause ^{15}N enrichment of residual N (Högberg, 1990; Högberg et al., 1995). Elevated emission of SO_2 and NO_x in Guiyang city have become important contributors to acid rain (Xiao et al., 2013), and thus, ammonia volatilization should be the exclusive pathway for ^{15}N enrichment in soil. For most vascular plants, nitrate and ammonium assimilation occurs mainly in the roots (Seith et al., 1994), and these N forms were preferentially absorbed by roots compared to N in the organic layer (Gebauer and Schulze, 1991). In addition, the pool and flux of amino acids in plants play an important role in N transport and proteins synthesis (Nordin et al., 2001). Through determination of $\delta^{15}N$ in amino acids, Gauthier et al. (2013) also found that the high accumulation or export flux of amino acids caused considerable isotope fractionation in leaves. After N compounds enter into plants, isotope fractionation caused by amino acids biosynthesis is approximately 17‰ (Yoneyama et al., 1993). Similar N isotope fractionation may tend to decrease $\delta^{15}N$ values, and thus assimilation and translocation processes of N within the plant could cause relatively ^{15}N-depleted N products compared with the original soil N source. Therefore, when Masson pine roots absorbed nitrate and ammonium in the soil (residual N with a high ^{15}N abundance) and these N were transported into leaves, the isotopic fractionation caused by N assimilation and translocation processes could lead to more positive $\delta^{15}N$ values in soils than in needles.

N uptake and translocation occurred at the roots or mycorrhiza
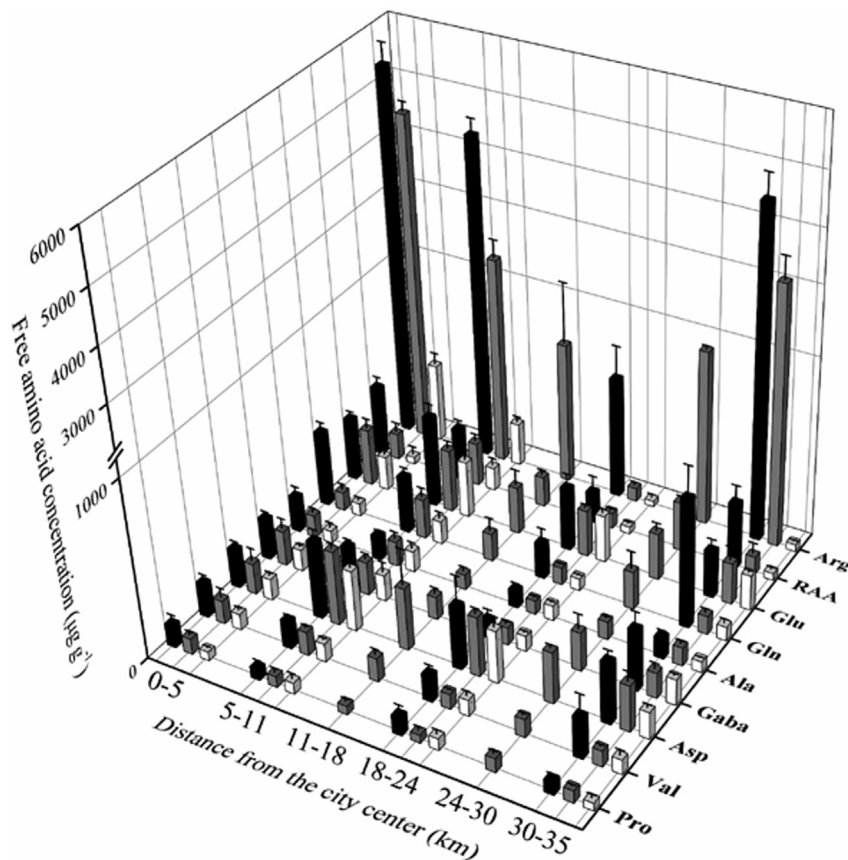
Fig. 4. Concentrations of major free amino acids in needles from central Guiyang to rural area. Black columns represent new current-year needles (new), gray columns represent mature current-year needles (middle-aged), white columns represent previous-year needles (old). (RAA-remaining amino acids). The vertical lines represent standard deviations.
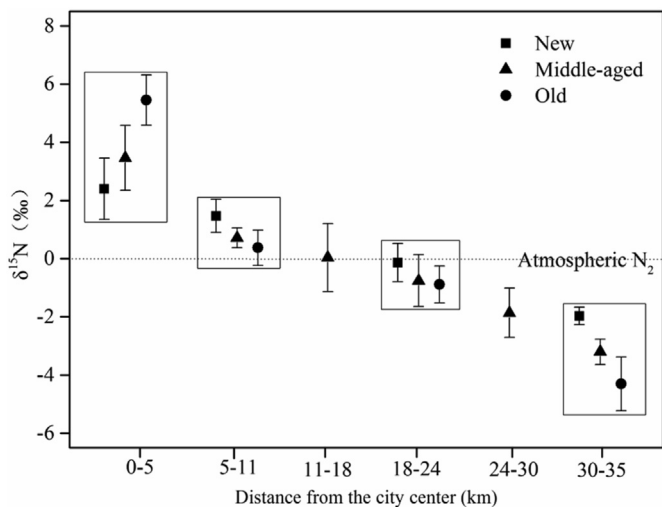
Fig. 5. Variations of $\delta^{15}N$ values in new, middle-aged and old needles from central Guiyang to rural area. The vertical lines represent standard deviations.

only cause very small N isotope fractionation when N is limited (Michelsen et al., 1996; Evans, 2001), so leaf $\delta^{15}N$ values probably reflect the $\delta^{15}N$ of soil N sources in N-limited ecosystems. However, it is not reasonable to use only leaf $\delta^{15}N$ values to reflect $\delta^{15}N$ of soil N sources in urban ecosystems. Isotopic discrimination during N uptake by plants will happen in most urban ecosystems with excess N supply (Evans, 2001). Moreover, factors such as atmospheric N,

root depth, soil N availability and the assimilation and translocation processes of N within the plant could affect $\delta^{15}N$ values in soil-grown plant leaves (Backéus, 1990; Högberg, 1997; Emmerton et al., 2001; Asada et al., 2005). Although foliar $\delta^{15}N$ of soil-grown plants could be affected by the above factors, some studies have suggested that the $\delta^{15}N$ values in soil-grown plant leaves could be linked to atmospheric N sources, namely, that foliar $\delta^{15}N$ values of plants grown near emission sources reflect the $\delta^{15}N$ values of the atmospheric N pollutant (Jung et al., 1997; Stewart et al., 2002). This study may provide further evidence that the $\delta^{15}N$ values in soil-grown plant leaves are useful indicators of atmospheric N deposition, as discussed below.

Firstly, compared to the strongly decreased trend in needle $\delta^{15}N$ values from urban to rural area, the decrease of soil $\delta^{15}N$ values was not significant ($P > 0.05$), and thus the difference in soil and needle $\delta^{15}N$ values (especially for old needles) increased significantly with distance. As discussed above, when soil N was transported into leaves, the isotopic fractionation caused by N assimilation and translocation within the plant led to more positive N isotopic values in soil than in leaves. However, these processes cannot result in the pronounced variations between needle $\delta^{15}N$ values in different areas, since soil $\delta^{15}N$ values were somewhat similar in this study. Moreover, because the leaves cannot prevent a passive uptake of substantial N compounds existed in the gas phase, in aerosols, and in precipitation, the $\delta^{15}N$ values in old needles should increasingly be affected by the $\delta^{15}N$ value of atmospheric N with exposure time, in other words, the needle $\delta^{15}N$ should increase with increasing leaf age if N uptake from atmospheric deposition were ^{15}N-enriched (Gebauer et al., 1994; Ammann et al., 1999). Although N would be

transferred from old leaves to the growing leaves following foliar abscission, the N isotope fractionation accompanying this process was small (Garten, 1993). Therefore, the only possible reason for the difference in soil and needle $\delta^{15}N$ values (especially for old needles) to increase with increasing distance is the influence of atmospheric N deposition. This result indicates that there are different sources for N deposition in urban and rural area.

Secondly, significantly positive $\delta^{15}N$ values were found in needles collected from the 0—5 km area (receiving the highest rates traffic-derived NO_x), and needle $\delta^{15}N$ values increased with needle age. However, ^{15}N-depleted needles were found in the rural area (30—35 km) and decreased with needle age (Fig. 5). Because atmospheric N compounds can be absorbed directly by leaves, foliar uptake of atmospheric N may exist with smaller isotope fractionation compared to the uptake of soil N by roots. Moreover, Masson pines of similar sizes and ages were chosen to reduce the influence of soil depth and canopy height (all branches were cut at similar heights) between sampling trees. Therefore, with the time of exposure, the $\delta^{15}N$ in old needles should be continuously affected by the $\delta^{15}N$ of atmospheric N pollutants, as mentioned above. The $\delta^{15}N$ values associated with NO_x-N from fossil fuel combustion are generally more positive than those associated with NH_x-N from agriculture and excretory waste. For example, Ammann et al. (1999) reported a traffic-derived NO_x isotopic composition of $+5.7 \pm 2.8‰$ for NO_2 and $+3.1 \pm 5.4‰$ for NO, and values for NO_x from coal combustion were $+6‰$ to $+13‰$ (Heaton, 1990), while the isotopic composition of NH_3 sourced from agricultural activities and animal excrement were $-5‰$ to $0‰$ and $-15‰$ to $-4‰$, respectively (Freyer, 1978; Heaton, 1987). In addition, Xiao and Liu (2002) reported a more positive signature of $\delta^{15}N$ for $-NO_3^-$ ($+2.0 \pm 4.4‰$) than for NH_4^+ ($-12.2 \pm 6.7‰$) in the rainwater of Guiyang. For Guiyang city, continuous development of the regional economy in recent years has contributed to rapidly increasing emission of NO_x (Tian et al., 2013). The average atmospheric NO_2 concentrations of 34.9 $\mu g\ m^{-3}$ in 2010 (Tian et al., 2013) and 40.2 $\mu g\ m^{-3}$ in 2016 (April 4th-June 15th) (data collected from http://www.ghb.gov.cn/kqzl.html.) in the urban center were well above the critical level of 10—15 $\mu g\ m^{-3}$ for NO_2 in the northern coniferous forests (Manninen and Huttunen, 2000) and the background value of 17.3 $\mu g\ m^{-3}$ for NO_2 in clean area of London (Carslaw and Carslaw, 2007). However, enhanced agricultural activities and continued population growth (increased excretory waste) have led to enormous NH_3 emission in rural areas (Liu et al., 2008). Based on the study of Xiao et al. (2010b), the total emission of NH_3 in Guiyang city in 2006 was about 72.6 kt (99.9% are from the anthropogenic NH_3 emission), of which the NH_3 emission from livestock accounted for 38.3%. In addition, N fertilizers applied in China were mainly urea (accounted for 35%) and NH_4HCO_3 (accounted for 56%), while more than half of the fertilizer N used in China (particularly in the Karst region) was lost to the environment in gaseous (e.g., N_2, NO and NH_3) or dissolved (e.g., $-NO_3^-$ and NH_4^+) forms, thus the extensive use of N fertilizers in rural areas can also contribute substantially to NH_3 emission (Cai et al., 1985; Zhu et al., 1989; Zhu and Chen, 2002). Thus, the above discussion indicates that we may attribute the age-related $\delta^{15}N$ values of needles in the 0—5 km area to a continuous uptake of ^{15}N-enriched atmospheric NO_x-N derived from traffic emissions. In contrast, in rural area (30—35 km), assimilation of isotopically light atmospheric NH_x-N resulted in ^{15}N-depleted needles, and $\delta^{15}N$ in needles decreased with increasing leaf age. Xiao et al. (2011) also observed that the $\delta^{15}N$ values in camphor leaves increased with leaf age near roads (high NO_x emission), but a decrease with leaf age occurred in camphor leaves near an industrial area (high NH_3 emission).

Lastly, in the 5—24 km area, although the $\delta^{15}N$ values in needles also decreased with increasing leaf age and showed a decreasing gradient with increasing distance from the city center, the difference in new and old needle $\delta^{15}N$ was not significant compared to that between the city center and rural area. We assume that the uptake amounts of atmospheric N by old needles is U_{old} and by new needles is U_{new}. It is clear that the needles gained relatively little N requirements by foliar absorption of atmospheric N in the 5—24 km areas (lower N deposition) and a lower U_{old}/U_{new} ratio occurred in those areas, so that a relatively slight age trend in needle $\delta^{15}N$ was found in those areas. In contrast, greater uptake from ^{15}N-enriched atmospheric N by old needles (higher U_{old}/U_{new} ratio) occurred in the city center (higher N deposition), and greater uptake of ^{15}N-depleted atmospheric N by old needles (higher U_{old}/U_{new} ratio) would be expected in rural area (higher N deposition). Therefore, it is possible to attribute the observed changes in the $\delta^{15}N$ of needles of different ages to the influence of atmospheric N.

5. Conclusion

Concentrations of both tissue N and free amino acids in needles were found to decrease with needle age in this study. Free amino acids in new and middle-aged needles were more sensitive biomarkers for atmospheric N deposition than in old needles. Moreover, elevated N and free amino acid (especially Arg) concentrations in needles reported here indicate a higher level of atmospheric N deposition in this study area.

The difference in soil and needle $\delta^{15}N$ values (especially for old needles) increased significantly with distance from the city center, which may be closely linked to atmospheric N deposition. Needle $\delta^{15}N$ values showed a strong gradient, with more positive values in the city center (0—5 km) (especially in old needles) and more ^{15}N-depleted in the rural area (30—35 km) (especially in old needles). Furthermore, in 5—24 km area, the difference between new and old needle $\delta^{15}N$ was not significant compared to the city center and rural area. The above results can be attributed to greater uptake from ^{15}N-enriched, traffic-derived NO_x by leaves in the city center and greater uptake from ^{15}N-depleted, agriculture-derived NH_x by leaves in the rural area, indicating that age-dependent needle $\delta^{15}N$ values could be better used to indicate atmospheric N sources. Therefore, this study provides new evidence that free amino acids and $\delta^{15}N$ values in needles are useful indicators of atmospheric N deposition.

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