Original Articles

Variations in free amino acid concentrations in mosses and different parts of *Cinnamomum camphora* along an urban-to-rural gradient

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ABSTRACT

Vegetation growing in urban ecosystems is frequently exposed to an environment with high atmospheric nitrogen (N) pollution. We systematically investigated the free amino acid concentrations in moss samples and *Cinnamomum camphora* leaf (new, middle-aged and old leaves), branch phloem, trunk phloem, and bark samples from field sites (Guiyang City, SW China) with different N deposition levels. The responses of the free amino acids to N deposition were analysed in the abovementioned plant tissues to determine whether plant free amino acids could act as biomarkers of the external N supply; moreover, differences in the N metabolism of these tissues under varying N deposition conditions were revealed by the compositions and sizes of their free amino acid pools. In particular, we reported the significant accumulation of arginine with increased N deposition in bark samples (while the arginine concentrations in the branch phloem, trunk phloem, and leaves remained low), which may indicate a long-term or historic external environment with a high N availability; additionally, the noticeable dominance and fluctuation of γ-aminobutyric acid in response to varied N deposition levels occurred in both the branch phloem and the trunk phloem, suggesting that the γ-aminobutyric acid transported in the phloem may be used as an important signal reflecting increases in the atmospheric N input. We conclude that the free amino acid concentrations in moss and camphor leaf, phloem and bark tissues are more sensitive to N deposition compared to their N concentrations and that tissue glutamine/glutamate and arginine/γ-aminobutyric acid ratios may serve as better biomarkers reflecting the tissue N accumulation status associated with increased N deposition. Therefore, free amino acid concentration analyses of different plant parts may provide a means to gain a more in-depth understanding of the impacts of atmospheric N pollution on plant physiology and N cycles.

1. Introduction

High anthropogenic emissions of reactive N have detrimental effects on the atmospheric quality and the health of terrestrial and aquatic ecosystems (Menon et al., 2007; Clark and Tilman 2008; Hastings et al., 2013; van Zanten et al., 2017). In contrast, N compounds from anthropogenic pathways also provide a significant input of available N via wet and dry deposition to soil and plants, which has received particular attention due to its potentially positive effects on plant growth (Townsend et al., 1996; Aber and Driscoll, 1997; Norby, 1998; Bauer et al., 2004). It is well known that the N demand of vascular plants is partly met by the root uptake of ammonium and nitrate with associated microbial processes, soil properties and natural or artificial factors (Magill et al., 2000; Mo et al., 2008; Cernusak et al., 2009; Xuan et al., 2017). If the available soil N is insufficient, the remobilization of N from internal plant resources can supply most N nutrition required for the development of new tissues (Arthur et al., 1998; Cherbuy et al., 2001; Millard and Grelet, 2010). Particularly in the early spring, low soil temperature leads to a lack of N nutrition for plants (Millard et al., 2006; Li et al., 2016). Additionally, N deposited from the atmosphere (mainly NH_x-N, NO_x-N and organic N) can be substantially absorbed or absorbed by aboveground plant parts in gaseous, dissolved, or particulate forms (Schulz et al., 2001; Lockwood et al., 2008; Padgett et al., 2009; Varela et al., 2016; Xu et al., 2017), causing some terrestrial vegetation to be partly independent of the soil N supply. For example, a study conducted by Ammann et al. (1999) found that oxidized N compounds represented approximately 25% of the N nutrition contribution to potted Norway spruce needles after the plants were placed near a highway for 4 months. Increases in the needle N concentration and biomass were also observed in Scots pine exposed to an

environment with high gaseous NH_3 levels, corroborating that atmospheric NH_3 inputs can be used as exogenous N fertilizer to maintain plant growth (Pérez-Soba and Van der Eerden, 1993).

Soil N (nitrate, ammonium and some organic N compounds) assimilated by plant roots or remobilized N from storage tissues is transported to tissues with N requirements partly or even largely in the form of amino acids through the xylem and/or phloem (Caputo and Barneix, 1997; Gourieroux et al., 2016; Tegeder and Hammes, 2018). Xylem transport is primarily governed by the transpiration stream in plants, but phloem transport is largely dependent on the relative status of the tissue N requirements, with transport directed from N sources to N sinks; moreover, N nutrients, such as free amino acids, are interchangeable between two transport systems (Arthur et al., 1998). In addition, according to the results of isotope labelling experiments, the atmospheric N compounds absorbed by plant leaves have long been known to be first incorporated into free amino acids (Nussbaum et al., 1993; Weber et al., 1995; Padgett et al., 2009). Thus, the pools and fluxes of free amino acid-N in different parts of plants play a significant part in protein synthesis and tissue N transport.

Based on this critical role of amino acids in plant N metabolism, further studies have found that plants are impacted by and show changes in the composition and size of their amino acid pools in response to different environmental stresses (e.g., water, mineral nutrient, heavy metal, N addition, pest damage, pathogens, temperature and salt stress); during these studies, arginine, γ-aminobutyric acid, asparagine, glutamine, glutamate, aspartate and proline have attracted close attention because of their significant accumulations (Chiozza et al., 2010; Liu et al., 2011; Garde-Cerdán et al., 2014; Hammad and Ali, 2014; Pavlíková et al., 2014; Postles et al., 2016). Such changes can thus be used as signals for indicating varied environmental conditions and the influences of environmental stress on plant N metabolism. Although previous studies have provided a large amount of information with regard to the potential effects of environmental stresses on the free amino acid concentrations of plant tissues, most of the experiments were performed in laboratories by simulating various environmental stresses or in natural habitats by the anthropogenic addition of the target substance (particularly simulated N deposition via fertilization). Furthermore, these earlier studies mainly focused on the mean concentrations of free amino acids in whole leaves; however, the determination of whole leaf results fails to consider differences in the free amino acid concentrations of leaves of different ages. In comparison, while a few studies investigating the responses of free amino acids in vascular plant leaves to changes in atmospheric N deposition (Power and Collins, 2010), studies on N deposition-induced variations in free amino acid concentrations in different plant parts (e.g., new, mature and old leaves, branch phloem and trunk phloem) are still limited, especially in the actual field environment. To date, several studies have fully evaluated the correlation between the chemical compositions of bark (outermost layer of the tree trunk) and atmospheric pollutants in an effort to find a proxy for the atmospheric pollution status (Schulz et al., 1997; Saarela et al., 2005; Kwak et al., 2009; Boltersdorf et al., 2014). These chemical indicators in the bark mainly included the $\delta^{15}N$, $\delta^{13}C$, N concentration, NO_3^- and NH_4^+ concentrations, and metal element concentrations. However, variations in the free amino acid concentrations of bark from sites with different atmospheric N pollution conditions have not been studied in detail, and their ability to record environmental N changes needs to be further confirmed.

Increasingly, analyses determining the positive and negative environmental effects of N pollution have focused on how N deposition impacts plant tissue nutrient N budgets and tissue N chemistry (Koranda et al., 2006; Padgett et al., 2009). To determine how the absorption and transport of N responds to the requirements of N-consuming tissues under different atmospheric N deposition levels, a signal for identifying the N status of plant tissues is needed; this signal can also be used as an indicator of increases in the atmospheric N input (Huhn and Schulz, 1996; Pitcairn et al., 2003). It has been suggested that the transport of amino compounds from the shoot to the roots through the phloem can reflect the plant N status (Weber et al., 1998). Such a signal associated with amino acid cycling between the N supply tissues and the N demand tissues also has critical roles in the regulation of the external N absorption by plants, such that the accumulated amino acid pools of these processes indicate both the external N supply and the internal N status of plants (Muller et al., 1996; Arthur et al., 1998). This proposal has been supported by experiments about the influence of anthropogenic N additions on the metabolism of N in conifers (Nordin et al., 2001). However, we need to extend this analysis to examine the consequences of high atmospheric N deposition (rather than N addition) for amino acid allocation and cycle in plants.

In this study, we analysed the variations in the free amino acid pools of mosses and different parts of camphor trees (new, middle-aged and old leaves, branch phloem, trunk phloem and the outermost bark) from field sites with different atmospheric N inputs. *Cinnamomum camphora* was selected for the study because it is widely distributed in Guiyang City as a main species used in urban greening. It is known that bryophytes primarily rely on atmospheric N to maintain their N requirements (Wilson et al., 2009; Skudnik et al., 2015). Based on this unique physiological characteristic of bryophytes, they are regarded as an excellent contrastive material to reveal the variability in the free amino acid pools in different parts of the camphor trees grown in the different field sites. The principal objectives of the present study were to determine which amino compounds are important indicators of the N nutrition status of the investigated plants and whether sensitive parameters exist in plants exposed to high atmospheric N inputs; furthermore, if these parameters do exist, what are the "dose-effect" relationships of N influence?

2. Materials and methods

2.1. Site characteristic

This study was conducted in Guiyang City (SW China) in a wide karst valley basin. The dense population and transportation network and rapidly developing industrial production and agricultural activities have made the city suffer serious environmental pollution (Tian et al., 2013; Liu et al., 2017). Sampling sites were chosen in the urban center (0–6 km), semi-urban areas (6–12 km area from the urban center), suburban areas (12–24 km area from the urban center) and rural areas (more than 24 km areas from the urban center) based on their different environmental characteristics (Fig. 1). The average total N deposition levels in the aforementioned areas were 29.21 ± 6.17, 21.98 ± 8.34, 11.64 ± 3.78, and 16.78 ± 4.32 kg N ha^{-1} yr^{-1}, respectively (Liu et al., 2009). This N deposition pattern, which decreases from the urban center to the suburban areas, is consistent with observation of atmospheric NO_2 concentrations, with average levels of 39.1 μg m^{-3} in the urban center and 18.6 μg m^{-3} in the suburban areas (Xu et al., 2018). The soil type in all the study areas was the same, characterized by acidic yellow soil with a low base saturation and high aluminium concentrations (Bohan et al., 1997). In addition, the average N concentrations and the average $\delta^{15}N$ values of the soil samples collected from the rooting zone at a depth of 0–10 cm in each area were not significantly different (data not shown). All sampling sites were at least 60 m away from the nearest pollutant sources and main roads.

2.2. Collection and treatment of samples

Sampling was carried out in June 2016 in either sunny or cloudy weather. New, middle-aged and old leaf, branch phloem, trunk phloem and outermost trunk (bark) samples from camphor trees approximately 8 m in height and approximately 15 years old were collected at 60 sites across the urban to rural gradient in Guiyang City (Fig. 1). Approximately 4–6 g of new current-year leaves (new, verdant leaves at the shoot apex with surface areas less than 10 cm^2), mature current-year
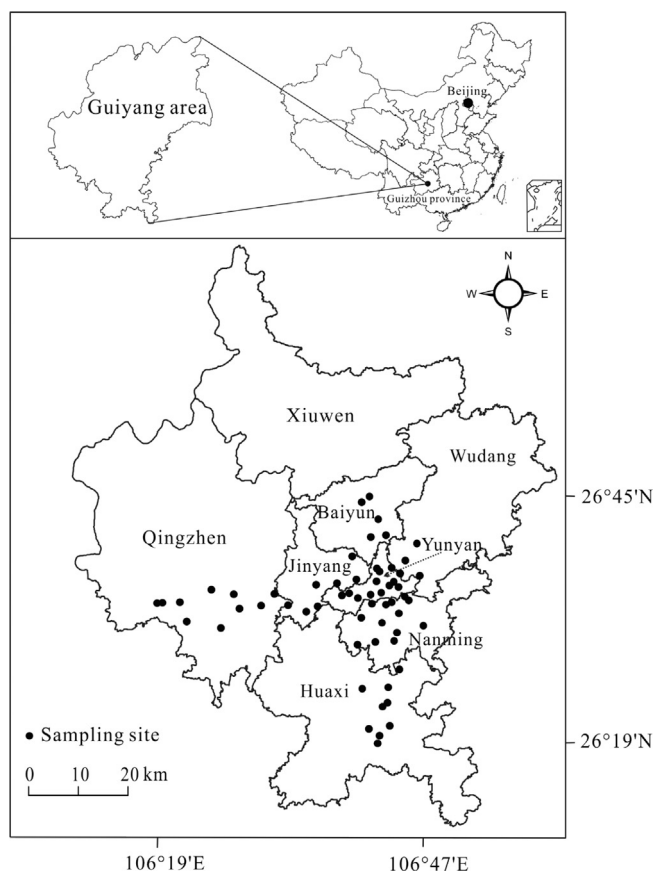
Fig. 1. Map showing the location of the Guiyang area and plant sampling sites.

leaves (middle-aged, 10–30 cm^2 surface areas) and previous-year leaves (old, non-verdant leaves grown in the lower layers) were sampled from the outer branches facing the north, west, south and east, at a height over 6 m above the ground. Approximately 10 mm and 1 cm of the external layers of the main branches (beneath each collected leaf sample) and the external layer of each trunk (approximately 1.5 m above the ground), respectively, were scraped off with a drawknife. One to three representative samples for each type of material were collected at each site. Concurrent to the collection of samples from different parts of the camphor trees, three replicate moss samples (mainly *Haplocladium microphyllum*) were also collected from bare rocks without coverage near the selected trees. All plant samples were immediately frozen in a car refrigerator after collection and then transported to the laboratory.

After the field work, all plant samples were cleaned using deionized water (Milli-Q). The outermost layer of each trunk sample was trimmed to approximately 2 mm thickness to serve as the bark material. Similarly, the middle part of the phloem from each trunk and branch sample was trimmed to approximately 2 mm thickness to serve as the trunk phloem and branch phloem material. Subsequently, all samples were freeze-dried and homogenized. All plant samples were stored at −80 °C until analysis.

2.3. Analysis of N and amino acid concentrations

Total N concentrations (%; dry weight) in samples were determined via a vario MACRO cube elemental analyser (Elementar, Frankfurt, Germany). The analytical precision of the N concentration measurement was 0.02% (n = 3). Subsamples comprising 0.05–0.25 g of each powder sample were used for the extraction of free amino acids. Free amino acids were extracted twice with TFA solution (6%; v/v), as described by Xu and Xiao (2017). The purified and concentrated amino

acid extraction solutions were then analysed directly by the Agilent 1260 series high-performance liquid chromatography system (Agilent Technologies, Palo Alto, USA) following the method of Xu and Xiao (2017). The detectable free amino acids in these plant samples included glutamate (Glu), aspartate (Asp), asparagine (Asn), glutamine (Gln), serine (Ser), tryptophan (Trp), lysine (Lys), isoleucine (Ile), leucine (Leu), arginine (Arg), citrulline (Cit), ornithine (Orn), alanine (Ala), glycine (Gly), threonine (Thr), proline (Pro), phenylalanine (Phe), methionine (Met), valine (Val), histidine (His), tyrosine (Tyr), and γ-aminobutyric acid (GABA).

2.4. Statistics

Significant differences in the mean values of the sample groups were evaluated using one-way variance analysis with Tukey's HSD test (at the 95% confidence level). The SPSS 19.0 statistical program (SPSS Inc., Chicago, USA) was used to conduct all statistical analyses, and all graphs were created via the Origin 9.0 software package (OriginLab Inc., Massachusetts, USA).

3. Results

3.1. N concentrations in mosses and different parts of the camphor trees

Mosses, new leaves, middle-aged leaves, old leaves, branch phloem, trunk phloem, and bark from the urban center showed the highest average N concentrations (Fig. 2). The average N concentrations in these plant samples from the suburban areas decreased by 6–37% compared to those from the urban center, with relatively small changes in the old leaves (6%) and new leaves (11%) and a reduction of up to 37% in mosses. In contrast with the significant decrease ($P < 0.05$) in the N concentration of each type of plant sample (except for new and old leaves) from the urban center to the suburban areas, the N concentrations increased slightly from the suburban areas to the rural areas (Fig. 2). This pattern in the plant tissue N concentrations was similar to variations in N deposition at the different sampling areas. Among camphor samples from the same areas, the new leaves showed a higher average N concentration than the other types of samples, and the bark had the lowest average N concentration (Fig. 2).

3.2. Free amino acid patterns in mosses and different parts of camphor trees

The spatial patterns of some important free amino acids in different parts of the camphor trees is shown in Figs. 3 and S1. The free Asp, Glu, GABA, Val, Ala, Gln, Pro, Asn, Ser, and Arg in the camphor tree tissues contributed more than 89% of the total free amino acid-N concentrations in all areas. Other amino acids, such as Trp, Lys, Ile, Leu, Cit, Orn, Gly, Thr, Phe, Met, His, and Tyr, were found in relatively small amounts in most of the samples. The main free amino acids in the new and middle-aged leaves were Asp, Glu, GABA, Val and Asn; in contrast, while these amino acids were also predominant in the old leaves, the concentrations of Asn was decreased by approximately 3–18 times. In the branch phloem and trunk phloem, GABA, Asp, Ala and Val represented the main amino acids. Differently, the strong accumulations of GABA, Asp, Ala and Val were not found in the bark, and the Arg concentrations in the bark were approximately 3- to 22-fold higher than those in the other tissues. In summary, the camphor trees exposed to high levels of atmospheric N deposition (the average level of total N deposition in each area is shown in the site characteristics section) mainly accumulated N in the form of Asp, Glu, GABA, Val and Asn in leaves and in the form of Asp, GABA, Ala and Val in the branch and trunk phloem, whereas the most abundant free amino acid in bark was Arg, followed by Gln and Glu. Obvious differences in the composition of the free amino acid pool between the camphor leaf tissues and the phloem and bark tissues were also revealed by the molar ratios of some important amino acids. For example, the Gln/Glu ratios of the branch
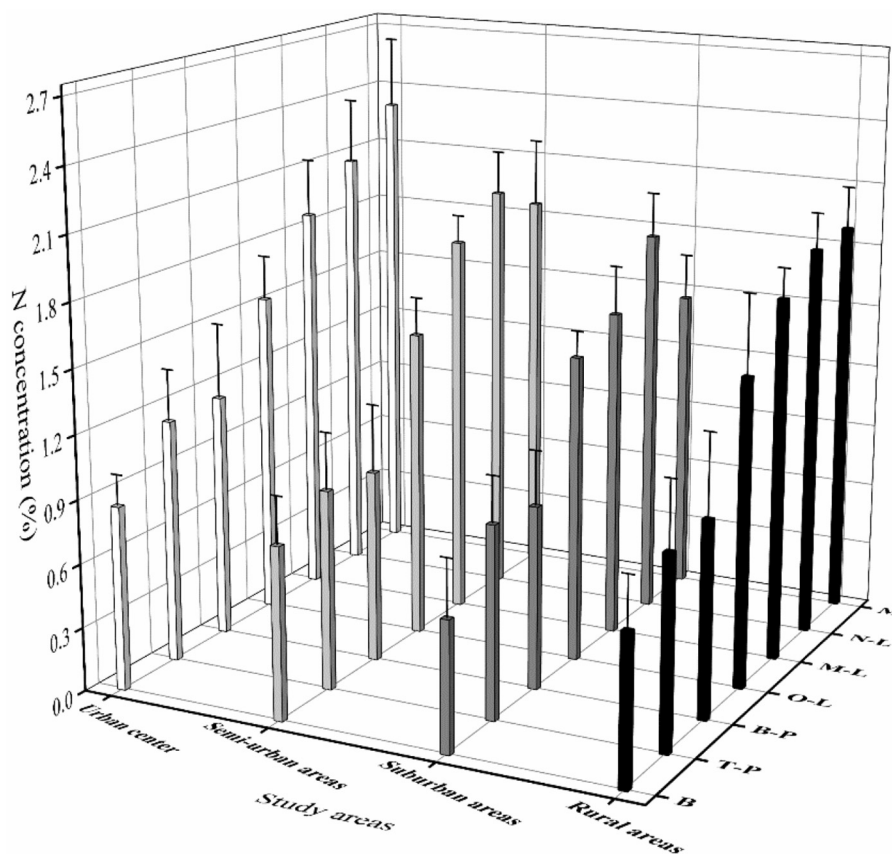
Fig. 2. Variations in N concentrations of new leaves (N-L), middle-aged leaves (M-L), old leaves (O-L), branch phloem (B-P), trunk phloem (T-P) and the outermost bark (B) of camphor trees and mosses (M) from different areas. The vertical lines represent standard deviations.

and trunk phloem and the bark were significantly higher than those of the leaf tissues; the bark Arg/GABA ratios demonstrated the highest growth, with levels that were 38- to 130-fold higher than those of the other tissues (Table. 1). For mosses, the main free amino acids were Arg, Asp, Glu, GABA, Ala, Gln, and Asn (Figs. 4 and S2). These free amino acids comprised 84–96% of the free amino acid-N pools in all areas. In particular, the moss Arg concentrations were the most variable, showing a strong accumulation in areas receiving high atmospheric N inputs (e.g., the urban center sites).

The variations in the total free amino acid, GABA, Asp, Glu, Gln, Arg, Asn, Ser and Val concentrations of the camphor leaves were consistent with the N concentration pattern of middle-aged leaves across the urban to rural gradient; moreover, at different areas, the variations in these amino acid concentrations were greater than the variations in the N concentrations (Figs. 2 and 3). However, the Ala and Pro concentrations exhibited no significant responses to the leaf N accumulation. The GABA concentration patterns in the branch phloem and trunk phloem and the Arg concentrations in the bark clearly resembled the N deposition gradient across the different areas. Across the study areas, the N deposition pattern was also reflected by changes in the moss total free amino acid, Arg, Ser, Ala, GABA, Glu, Asp, Asn and Gln concentrations. When comparing the Gln/Glu ratios in samples from different areas, samples in the urban center had relatively higher Gln/Glu ratios than those in the suburban areas. A similar decreasing trend from the urban center to the suburban areas was also observed for the sample Arg/GABA ratios (Table. 1).

4. Discussion

Similar to relatively fragile terrestrial ecosystems, such as heaths, bogs and some coniferous forests (the critical N load of less than

$10 \, \text{N} \, \text{ha}^{-1} \, \text{yr}^{-1}$) (Manninen and Huttunen, 2000; Krupa, 2003), the capacity to assimilate and retain N in urban vegetation is also finite. If the external N availability is supraoptimal, plants detoxify and store surplus N primarily as a soluble amino acid-N fraction (Calanni et al., 1999). As mentioned in the introduction, N deposition contributes to part of the N nutrition of plants. Once atmospheric N enters leaf tissues, nitrate/nitrite reductase pathways are used; these external N sources (e.g., NO_x-N and NH_x-N) are subsequently assimilated into amino acids via the Gln-Glu cycle (Fig. 5). A rapid amino acid response to N deposition is thus expected based on the described processes. High N inputs have previously been found to change the compositions of N compounds in conifer, bryophyte, oak, and some herbaceous plant leaves (Koranda et al., 2006; Limpens and Berendse, 2003; Näsholm et al., 1994; Padgett et al., 2009), such that a healthy plant N status could be maintained. Typically, the Arg (C/N = 1.5) demonstrates the greatest proportional increase in non-protein N concentrations, which represents an economical and highly efficient strategy for tissue N metabolism (Huhn and Schulz, 1996), followed by increases in Gln (C/N = 2.5) and Asn (C/N = 2.0). In this study, moss Arg increased notably in response to high atmospheric N inputs (at the urban center). However, for the camphor leaves, branch phloem and trunk phloem, a different N translocation mechanism appeared to operate, as the free amino acid-N in these tissues primarily accumulated as GABA, Asp and Val rather than Arg (Figs. 3 and S1). One possible cause for the lack of an Arg response in camphor leaf, branch phloem and trunk phloem tissues is that tissue Arg is rapidly used for protein synthesis or transformed into other amino acids (e.g., GABA) (Fig. 5). In this case, GABA functions to temporarily store N as a means of recycling Arg-derived C and N (Bown and Shelp, 1997). However, in plants, GABA is frequently treated as a metabolite or signal molecule, mainly in respect to plant responses to oxidative stress, water and salt stresses, insect and
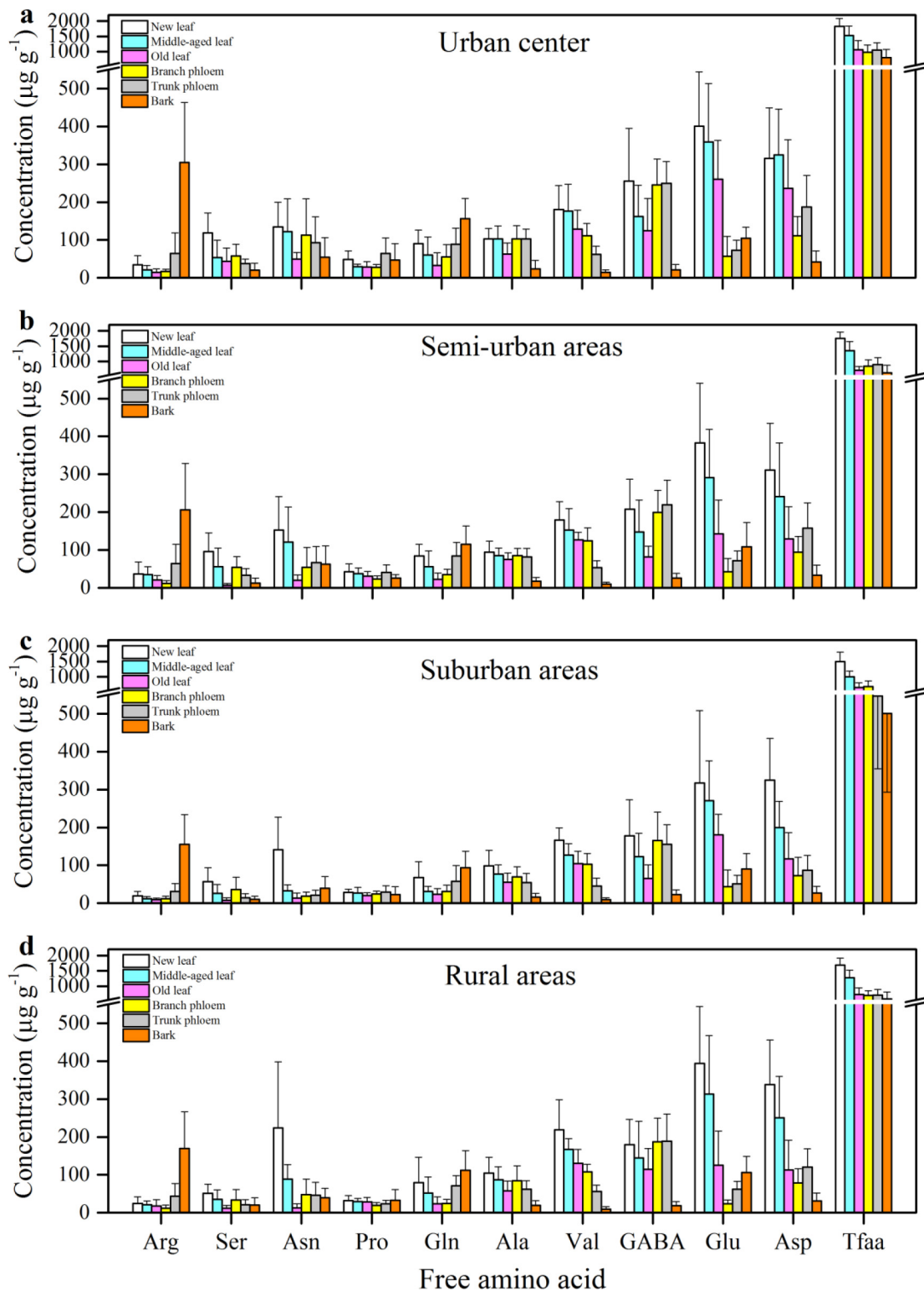
Fig. 3. Variations in major free amino acid concentrations (Tfaa: total free amino acid) of new leaves, middle-aged leaves, old leaves, branch phloem, trunk phloem and bark of camphor trees from the urban center (a), semi-urban areas (b), suburban areas (c) and rural areas (d). The break in the Y axis is set at 550–560 µg g^{-1} (dry weight). The vertical lines represent standard deviations.

pathogen attacks, high and low temperatures, anoxia, and mechanical stimulation (Kinnersley and Turano, 2000; Bown et al., 2002; Bouché and Fromm, 2004; Bown et al., 2006; Liao et al., 2017). Previous studies investigating free amino acid compositions as indicators of N deposition have mainly focused on the accumulation of Arg, Gln, Glu, Asn and Asp in needles (Schneider et al., 1996; Calanni et al., 1999; Nordin

et al., 2001). In these studies, GABA was ignored or not detected. Actually, GABA in plants can be rapidly and highly produced in response to high N deposition. As in this study, the GABA concentrations in mosses and camphor leaves of different ages can reach 33–91% increases from the suburban area to the urban center (Figs. 3 and 4). Considering the lack of a significant response by camphor leaf Arg (low

Table 1
The Gln/Glu and Arg/GABA molar ratios (mean ± SD) in mosses and different parts of camphor trees from different sampling areas.

		Study areas			
		Urban center	Semi-urban areas	Suburban areas	Rural areas
New leaf	Gln/Glu	0.27 ± 0.15 (a)	0.28 ± 0.16 (a)	0.23 ± 0.16 (a)	0.27 ± 0.19 (a)
	Arg/GABA	0.10 ± 0.07 (A)	0.08 ± 0.05 (A)	0.07 ± 0.04 (A)	0.09 ± 0.06 (A)
Middle-aged leaf	Gln/Glu	0.25 ± 0.17 (a)	0.23 ± 0.16 (a)	0.11 ± 0.06 (b)	0.24 ± 0.19 (a)
	Arg/GABA	0.15 ± 0.10 (A)	0.10 ± 0.05 (A)	0.10 ± 0.07 (A)	0.13 ± 0.09 (A)
Old leaf	Gln/Glu	0.15 ± 0.12 (ab)	0.15 ± 0.06 (ab)	0.07 ± 0.04 (b)	0.23 ± 0.14 (a)
	Arg/GABA	0.17 ± 0.10 (A)	0.14 ± 0.07 (A)	0.13 ± 0.09 (A)	0.15 ± 0.13 (A)
Branch phloem	Gln/Glu	1.67 ± 1.32 (a)	1.22 ± 0.88 (a)	1.18 ± 0.79 (a)	1.24 ± 0.69 (a)
	Arg/GABA	0.06 ± 0.03 (A)	0.04 ± 0.02 (AB)	0.03 ± 0.01 (B)	0.05 ± 0.03 (AB)
Trunk phloem	Gln/Glu	1.31 ± 0.59 (a)	1.24 ± 0.49 (a)	1.21 ± 0.65 (a)	1.22 ± 0.42 (a)
	Arg/GABA	0.20 ± 0.14 (A)	0.14 ± 0.08 (A)	0.12 ± 0.07 (A)	0.15 ± 0.11 (A)
Bark	Gln/Glu	1.52 ± 0.44 (a)	1.39 ± 0.69 (a)	1.20 ± 0.68 (a)	1.33 ± 0.70 (a)
	Arg/GABA	13.07 ± 9.40 (A)	6.41 ± 3.13 (AB)	4.90 ± 2.37 (B)	6.58 ± 2.94 (AB)
Moss	Gln/Glu	0.55 ± 0.16 (a)	0.48 ± 0.23 (ab)	0.36 ± 0.17 (b)	0.50 ± 0.16 (ab)
	Arg/GABA	4.65 ± 2.90 (A)	3.90 ± 2.66 (AB)	2.05 ± 1.40 (B)	4.10 ± 2.37 (AB)

Different letters indicate a significant difference between the means of the sample groups (marked with lowercase letters for Gln/Glu ratios and uppercase letters for Arg/GABA ratios) ($P < 0.05$).
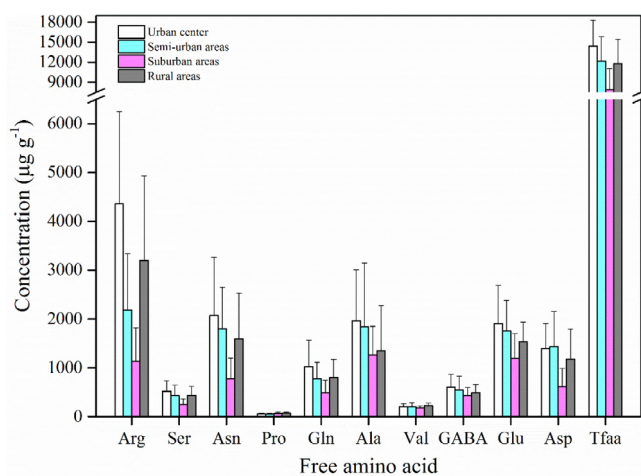

Fig. 4. Variations in major free amino acid concentrations (Tfaa: total free amino acid) of mosses from different areas. The break in the Y axis is set at 6500–7500 $\mu g\,g^{-1}$ (dry weight). The vertical lines represent standard deviations.

Fig. 5. The GABA and Arg metabolic pathways and their relationships to other metabolic pathways in plants (summarized from Shelp et al. (1999), Bhatnagar et al. (2001) and Deleu et al. (2013)). α-KG: α-Ketoglutarate. SSA: Succinic semialdehyde. Some key enzymes are: 1, nitrate reductase and nitrite reductase; 2, glutamine synthetase; 3, glutamate synthase; 4, glutamate decarboxylase; 5, pyruvate dependent GABA transaminase; 6, α-ketoglutarate dependent GABA transaminase; 7, succinic semialdehyde dehydrogenase; 8, glyoxylate reductase (SSA reductase); 9, Δ^1-pyrroline-5-carboxylate synthetase and Δ^1-pyrroline-5-carboxylate reductase; 10, proline dehydrogenase and Δ^1-pyrrolidine-5-carboxylate dehydrogenase; 11, arginine synthase; 12, arginine decarboxylase; 13, arginase; 14, diamine oxidase; 15, butyraldehyde dehydrogenase; 16, spermidine synthase; 17, polyamine oxidase; and 18, spermine synthase.

concentrations) to increased N deposition, GABA may thus assimilate C and N from Glu and Arg to participate in general N metabolism (Fig. 5). This finding revealed that GABA in camphor leaves can store N in place of Arg when the external N input is high.

Although there are several reports comparing the amino acid compositions among different parts of coniferous trees (e.g., needles, xylem and phloem) exposed to varied N deposition levels (Arthur et al., 1998; Weber et al., 1998), to date, no studies have attempted to analyse the effects of atmospheric N pollution on the amino acid pool in the outermost layer of the trunk (described as bark). In bark, Arg, Gln and Glu were the most abundant amino acids, together contributing to over 76% of the total free amino acid-N concentrations; other amino acids that were relatively enriched in the camphor leaf tissues, such as GABA, Asp and Val, were present in small amounts in the bark, together comprising less than 7% of the total free amino acid-N concentrations. In particular, it is interesting to note that highly accumulated Arg concentrations were recorded in the bark samples (up to 20-fold difference between the leaves and bark). Since bark, as the outermost layer of the trunk, does not have any metabolic activity, the high Arg concentrations cannot be derived from the instant delivery of supply tissues;
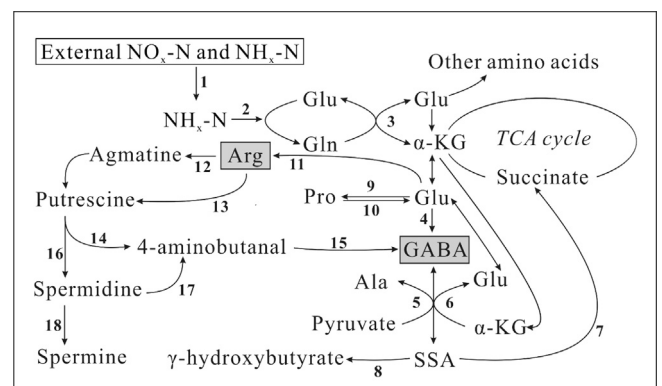
furthermore, although bark is regarded as an excellent passive adsorbent of environmental pollutants (Schulz et al., 1999), the fact that rainwater Arg concentrations were less than 0.15 $\mu mol\,L^{-1}$ (data not shown) makes it impossible for the bark to be substantially enriched by environmental Arg. Thus, it is reasonable to attribute the high Arg concentrations in the bark to the primary accumulation of Arg in the early plant development stage or the release of Arg during programmed cell death. In addition, the increase in bark Arg concentrations from the suburban areas to the urban center was found. These results suggested that, at least for camphor tress, the substantially retained Arg in bark in the urban center may reflect a long-term or historic environment of high N availability in this area.

According to the above discussion, it is accepted that the amino acid cycle in plants is a complicated process with individual and organ differences. However, free amino acid concentrations in plant leaf tissues have been found to be tightly correlated with variations in atmospheric

N deposition. As previously reported for bryophytes, significantly positive correlations were observed between the free Arg concentrations in *Rhytidiadelphus triquetrus* ($R^2 = 0.970$), *Brachythecium rutabulum* ($R^2 = 0.899$) and *Pseudoscleropodium purum* ($R^2 = 0.780$) and the log distance from an atmospheric NH_x-N source (poultry farm) (Pitcairn et al., 2003). For vascular plants, N and free amino acid concentrations can be affected by soil N availability but also show a significant relationship with increasing atmospheric N inputs. For example, Power and Collins (2010) detected signals of N deposition in the London area by using the free amino acids in *Calluna vulgaris* leaves as biomarkers, finding that foliar free Phe ($R^2 = 0.774$; $P < 0.0001$), Gln ($R^2 = 0.661$; $P < 0.0001$), Gly ($R^2 = 0.528$; $P < 0.001$), Asn ($R^2 = 0.438$; $P < 0.01$), Ile ($R^2 = 0.336$; $P < 0.0001$) and Lue ($R^2 = 0.293$; $P < 0.001$) concentrations significantly responded to variations in the atmospheric N deposition. While these limited studies demonstrated that fluctuations in the free amino acid concentrations of plant tissues can be used to assess atmospheric N deposition, we expected to reveal the sensitivity and reliability of plant tissue free amino acids as biomarkers for atmospheric N pollution from another perspective. An increase in the external N uptake would directly cause an increase in the Gln/Glu ratio due to the increased ammonium utilized in the Glu-Gln cycle (Fig. 5). Likewise, the Arg/GABA ratio represents increases in the accumulation of external N in plant tissues when excess N is stored or detoxified, mainly in the form of Arg or GABA. In addition, based on the similar climatic conditions and soil properties in these test areas, it can be concluded that the free amino acid pools in the plant tissues showed strong responses to the varied N deposition levels (Figs. 3 and 4). Accordingly, this response can be particularly well reflected in tissue Gln/Glu and Arg/GABA ratios. When comparing samples from different sampling areas, higher tissue Gln/Glu and Arg/GABA ratios (particularly in mosses, middle-aged leaves and bark) were observed in the urban center (Table. 1), corresponding to the higher total N deposition level in this area ($29.21 \pm 6.17 \, \mathrm{kg \, N \, ha^{-1} \, yr^{-1}}$); conversely, the tissue Gln/Glu and Arg/GABA ratios were lowest in the suburban areas, where the recorded N deposition was relatively low ($11.64 \pm 3.78 \, \mathrm{kg \, N \, ha^{-1} \, yr^{-1}}$). However, slightly elevated tissue Gln/Glu and Arg/GABA ratios reappeared in the rural areas, which may be explained by the fact that the agricultural activities with high NH_3 emissions in these areas increase the N deposition level ($16.78 \pm 4.32 \, \mathrm{kg \, N \, ha^{-1} \, yr^{-1}}$) (Liu et al., 2008). It is important to note that the Gln/Glu and Arg/GABA ratios in the new leaves, old leaves and phloem showed a relatively weak response to N deposition compared to those of the mosses, middle-aged leaves and bark. This may be attributed to the rapid protein synthesis in new leaves for growth, the positive remobilization of stored N from old leaves, and the frequent material exchange between xylem and phloem systems. For bryophytes, the atmospherically deposited N is the main N source used for growth. Moreover, we found that moss Arg showed the highest concentrations and variations within the sampling areas. Thus, the average Gln/Glu ratio of 0.38 and the average Arg/GABA ratio of 2.05 in mosses from the suburban areas (the lowest N deposition level) may serve as key values indicating a decrease in protein synthesis and an accumulation of Arg, an important intermediate in the amino acid cycle, to remove excess external Huhn and Schulz (1996) carried out a study about the effects of different N deposition levels on the free amino acid concentrations of needles, revealing that the Gln/Glu ratio rose after N inputs increased, and that the average Gln/Glu ratio of 0.36 was a critical value reflecting increased amino acid synthesis. As mentioned earlier, the processes transforming or translocating assimilated N in plant tissues may be species dependent. Unlike mosses, the composition and size of the free amino acid pool in vascular plants can be affected by soil N availability because amino acids can be transported into the aboveground parts of plants via the xylem system (Schneider et al., 1996). Additionally, atmospheric N absorbed by camphor leaves can be assimilated into organic N and then transferred to other tissues (e.g., roots) in the form of free amino acids, providing the precursors of

protein synthesis. Therefore, the composition and size of the free amino acid pool should be expected to differ between camphor trees and mosses, with relatively lower Gln/Glu and Arg/GABA ratio averages in camphor leaves than in mosses in each test area. By comparing the patterns between tissue N and free amino acid concentrations along the urban to rural gradient, we also observed that variations in the Gln/Glu and Arg/GABA ratios in the mosses, middle-aged leaves and bark from the urban center to the rural area were more significant than their N concentration variations. Thus, Gln/Glu and Arg/GABA ratios in mosses and the bark and middle-aged leaves of camphor trees can be used as sensitive and reliable biomarkers of atmospheric N deposition.

The composition and size of the free amino acid pool in different parts of the camphor trees not only reflected differences in the atmospheric N inputs, as discussed above, but also might provide additional information about the influence of high N deposition on the distribution and cycling of plant amino acid-N. High free amino acid concentrations were found in the new camphor leaves, while there was a high decrease in the free amino acid concentrations of the old camphor leaves (Fig. 3). This decrease can be primarily attributed to variations in the Glu, Asp, Asn, GABA and Val concentrations, the prevailing amino acids in the camphor leaves. It is obvious that the remobilization of N from storage tissues (e.g., older leaves) and the input of xylem amino acids are important sources of nutrition for the growth of newly developing tissues. Similarly, Millard and Grelet (2010) reported that N mobilized from old leaves contributes a proportion of the available N to new leaves. While examining the N status of beech and spruce, both Schneider et al. (1996) and Arthur et al. (1998) found that old leaves contained large quantities of easy-to-use amino acid-N, most of which was relocated to maintain the N requirements of new tissues, and that the xylem could transport amino acid-N into the leaves. In addition to the translocation of N inside plants, the relatively higher free amino acid levels detected in the new and mature (middle-aged) camphor leaves might have considerably originated from the assimilation of atmospheric N compounds (e.g., NO_x-N and NH_x-N) by leaf tissues. New and mature camphor leaves should have a highly active metabolic activity level for the assimilation of atmospheric N, while the senescent old leaves, in contrast, should exhibit the anabolic and catabolic decay of N compounds (Gebauer et al., 1994). Thus, although the old leaves had been theoretically accumulating deposited N for a longer time than younger leaves, the remobilization of the original stored N and the sluggish metabolism of older leaves would prevent the net accumulation of atmospheric N. The decrease in the Gln/Glu ratio from new leaves to old leaves also further supports the abovementioned deduction. Accordingly, the free amino acid concentrations in new and mature leaves may be more likely to accumulate in response to high N deposition levels.

Similar amino acids dominated the branch phloem and trunk phloem. However, the sizes of free amino acid pools in the branch phloem and trunk phloem were significantly lower than those in the new and middle-aged leaves. This difference was mainly caused by a dramatic decrease in the Asp and Glu concentrations of the branch phloem and trunk phloem. Additionally, Asp and Glu concentrations gradually increased from the branch phloem to the trunk phloem. Therefore, it can be inferred that Asp and Glu were either transported in the phloem to the tissues with N demands (e.g., the roots) in order to maintain a healthy plant N status or were reloaded into developing leaf tissues via the upward translocation of amino acids from the phloem to xylem. A study on the cycle of amino acids in the leaves, phloem and xylem of beech and spruce trees by Arthur et al. (1998) revealed that Asp and Gln play vital roles in the N cycle of beech and spruce trees, which further confirms the above results of the current study. GABA accounted for the largest portion of the free amino acid pool in both the branch phloem and trunk phloem and significantly increased from the leaves (except for the new leaves, among which the GABA concentrations had not strong fluctuation) to the phloem; moreover, a large increase in GABA in response to increased atmospheric N supply was also observed in the branch phloem and trunk phloem (Fig. 3). In contrast,

Glu and Arg, which are involved in GABA anabolism, were found in relatively small amounts in the phloem irrespective of test area variations (corresponding to different atmospheric N supplies). It is thus possible that the significant dominance and fluctuation of GABA in the phloem were directly correlated with atmospheric N inputs. This result also further indicates that the GABA transported in the phloem may be used as an important signal of increased N deposition.

5. Conclusions

In both camphor trees and mosses, free amino acid-N plays a crucial role in the internal metabolism of N and responses to external N inputs. We found that tissue Gln/Glu and Arg/GABA ratios may serve as sensitive biomarkers of N deposition that additionally reflect the tissue N accumulation status. Mosses, middle-aged leaves and bark in the urban center had the highest Gln/Glu and Arg/GABA ratios, clearly indicating the higher N deposition level in this area; subsequently, the Gln/Glu and Arg/GABA ratios in these tissues demonstrated the lowest values in the suburban areas where recorded a lower N deposition level. The slight increase in the tissue Gln/Glu and Arg/GABA ratios from the suburban to the rural areas may suggest an increase in N deposition associated with the enhanced NH_3 emissions from rural agricultural activities. Since atmospheric N inputs represent the main source of N nutrition for moss growth, we concluded that the average Gln/Glu ratio of 0.38 and the average Arg/GABA ratio of 2.05 in mosses from the suburban areas (with a N deposition level of $11.64 \pm 3.78 \, \text{kg N ha}^{-1} \text{yr}^{-1}$) may serve as crucial values reflecting increases in amino acid synthesis to remove excess external N.

In particular, compared to the low Arg concentrations in the branch phloem, trunk phloem, and all leaves, significantly accumulated Arg with increased N deposition occurred in the bark samples. It is therefore possible that the considerable levels of retained Arg in bark indicate long-term or historic external environments with high N availability. Additionally, the significant dominance and fluctuation of GABA in response to varied N inputs were observed in both the branch phloem and trunk phloem, accompanied by a significant increase from the old leaves to the middle-aged leaves and then to the phloem. Therefore, GABA transported in the phloem may serve as an important signal indicating increased N inputs. It was concluded that the transport, storage and remobilization of free amino acids in plants indicate the internal cycling of N and the external N supply, such that the N deposition levels and N nutrition status of plants can be assessed based on variations in free amino acid concentrations in plant tissues.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ecolind.2018.04.030.

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