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Amine distribution and content in several parts of the subantarctic endemic species *Lyallia kerguelensis* (Hectorellaceae)

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Abstract

The leaves, stems and roots of the subantarctic endemic species from Kerguelen *Lyallia kerguelensis* showed different patterns of amine accumulation. The leaves contained the highest free amine, stems the intermediate and roots the lowest content. Leaves accumulated massively agmatine and tyramine, whereas stems contained high levels of octopamine and spermidine. A high content of an amine which was co-chromatographed with homospermidine was observed in leaves and stems. Conjugated amines did not accumulate to any significant extent in stems and roots, whereas they were detected in high amounts in leaves.

Keywords: *Lyallia kerguelensis*; Hectorellaceae; Amine distribution; Aromatic amines; Polyamines; Conjugated amines; Ancestral

1. Introduction

Lyallia kerguelensis Hook. f. (Hectorellaceae) is an endemic species from Kerguelen Archipelago in the subantarctic. This cushion plant is generally less than 50 cm in diameter. The species has a rather limited distribution, restricted to small populations on stony barrens (Hennion & Walton, 1997). Kerguelen climate is characterized by a permanent cold temperature with 2°C in winter and 8°C in summer. However, a significant increase of mean annual temperature and decrease of precipitation level has been recorded over the past 40 years (Frenot, Gloaguen, & Trehen, 1997). These changes may strongly affect the life of species adapted to a cold climate with little seasonality. Amines (polyamines, aromatic monoamines) and the amine conjugates (especially hydroxycinnamoyl amine conjugates) may play an important role in many aspects of plant development including growth, differentiation, senescence and response to several environmental challenges (reviewed in Bouchereau, Aziz, Larher, & Martin-Tanguy (1999); Kumar, Altabella, Taylor, & Tiburcio (1997); Martin-Tanguy (1997)). We have started work on the accumulation of organic solutes in the endemic species from Kerguelen (Aubert, Assard, Boutin, Frenot, & Dorne, in press; Aubert et al., in press; Hennion & Bouchereau, 1998) but to our knowledge no work has been done on amine distribution and content in subantarctic species. Thus the present work aimed at determining the content and distribution of free amines (polyamines and aromatic amines) and their conjugates in the different parts from one of these species, *Lyallia kerguelensis*. This study is part of a continuing research program on the functioning and evolution of the subantarctic ecosystem at Kerguelen.

2. Results and discussion

The free polyamines present in *Lyallia* were agmatine, putrescine, spermidine, spermine, homospermidine, diaminopropane (an oxidation product of spermidine and/or spermine) and cadaverine (Fig. 1). We identified several types of aromatic monoamines. Their structures are described in Fig. 2. As shown in Table 1 agmatine (an intermediate product of putrescine synthesis via arginine decarboxylase activity in

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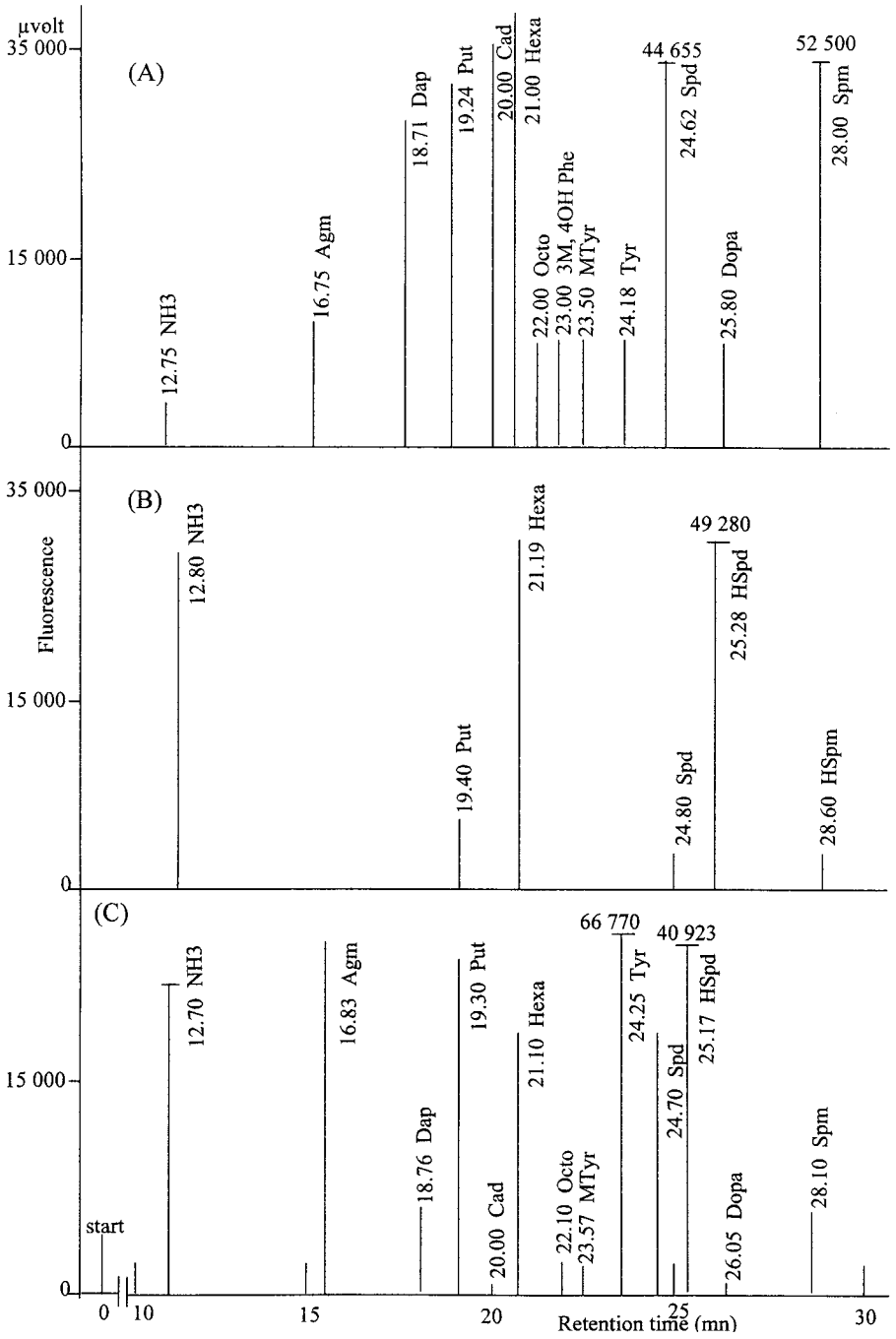
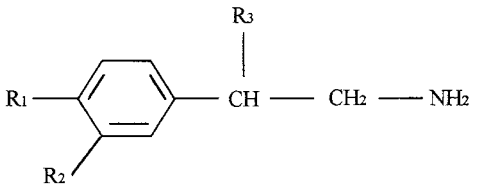


Fig. 1. Separation of dansylamines. (A) standards, 0.10 nmol each, (B) homospermidine from N_2-fixing eubacterium *Rhizobium*, (C) leaf extracts from *Lyallia kerguelensis*, by reverse phase HPLC on a 5 µm C18 ultrasphere column (4.6 × 250 mm, Beckman) using a gradient of methanol:-water (60–95% over 23 min) at a flow rate of 1 ml min^{-1}. $t_0 = 2.03$ min. Sample volume was 20 µl. Detection: excitation 365 nm, emission 510 nm. Hexanediamine was used as an internal standard. Agm, agmatine; Dap, diaminopropane; Put, putrescine; Cad, cadaverine; Hexa, hexanediamine; Octo, octopamine; 3M, 4OH Phe, 3-methoxy, 4-hydroxyphenethylamine; Tyr, tyramine; Spd, spermidine; HSpd, homospermidine; Dopa, dopamine; Spm, spermine; HSpm, homospermine.

higher plants) accumulated in the leaves of *Lyallia*. It constituted the major part of the free polyamine pool, namely 60% of the whole. Tyramine accumulated massively in these organs. It represented 90% of the total free aromatic amine pool and it made up about 50% of the total free amine content. In stems spermidine constituted the major part of the free polyamine pool, namely 33% of the whole, whereas octopamine represented 66% of the total free aromatic amine pool. An uncommon amine which was cochromatographed



R1	R2	R3	compound
OH	H	H	Tyramine
OH	OH	H	Dopamine
OCH_3	H	H	Methoxy-tyramine
OH	OCH_3	H	3-methoxy, 4-hydroxy phenethylamine
OH	H	OH	Octopamine

Fig. 2. Structures of aromatic monoamines identified in *Lyallia*.

in both TLC and HPLC with homospermidine was also detected. It was recorded in higher concentrations in leaves than in stems. The roots exhibited a low polyamine content (Table 1). The conjugated amines contained ferulic acid and amines linked by an amide bound. There were two types: basic amides with a primary amine function and neutral amides showing no strongly ionizable function (Table 1). The amines of basic amides included both di- and polyamines whereas those of neutral amides included both aliphatic and aromatic amines. Basic conjugates did not accumulate to any significant extent in the various parts of *Lyallia*. Neutral conjugates of polyamines and aromatic amines accumulated in leaves. Diferuloylspermidine constituted the major part of the neutral polyamine pool, namely 60% of the whole. Conjugated forms of aromatic amines, mainly tyramine and octopamine, dominated neutral polyamine conjugates by a factor of 3. In contrast, stems and roots exhibited a low neutral amide content (Table 1).

The massive accumulation of agmatine and the detection of the uncommon polyamine, homospermidine, in the leaves of *Lyallia* raise some questions. To our knowlegdge this is the first report of a massive accumulation of agmatine in the leaves of a non-stressed higher plant. Furthermore it has earlier been reported that higher plants subjected to various stressing conditions (osmotic stress, low temperature, ...) exhibited greater ADC activity, but no data have been presented concerning the involvement of its product, agmatine, in the reactions to stress. However Racz et al. (1996) have recently reported that agmatine, in wheat, may play an important role during a short cold treatment. The quantity of agmatine increased in leaves as the result of a cold treament at $-2°C$ and this trend showed a positive correlation with the frost resistance of the varieties: there was an increase in agmatine induction parallel to the increase in frost resistance. Thus, the possibility of agmatine playing a major role in cold-induced reactions merits further investigation.

The biochemistry and physiological significance of aromatic amines in plants are poorly understood. Tyramine and octopamine are antagonists to cytokinins (Bouchereau et al., 1999). Dopamine can inhibit IAA oxidation in vitro as well as in vivo (Protacio, Dai, Lewis, & Flores, 1991). Thus, the growth-promoting effect of catecholamines is due to their inhibition of IAA degradation, resulting in higher levels of auxin. Recently for the first time a conjugate of tyramine and jasmonic acid has been identified from *Petunia* pollen (Miersch, Knöfel, Kramell, & Parthier, 1998).

Table 1
Free and conjugated amine levels in several parts of *Lyallia kerguelensis*. Agm, agmatine; Put, putrescine; Spd, spermidine; HSpd, homospermidine; Spm, spermine; Dap, 1,3-diaminopropane; Cad, cadaverine; PAs, polyamines; Phe, phenethylamine; Tyr, tyramine; M-Tyr, methoxy-tyramine; Octo, octopamine; Dopa, dopamine; 3M, 4OH-Phe, 3-methoxy, 4-hydroxyphenethylamine; AAs, aromatic monoamines; –, not detected

	Free amines (nmol. g^{-1} dw)														
	polyamines								aromatic monoamines						
	Agm	Put	Spd	HSpd	Spm	Dap	Cad	Total PAs	Tyr	M–Tyr	Octo	Dopa	3M, 4OH–Phe	Total AAs	
Root	181	84	84	39	–	73	–	461	41	41	43	–	–	125	
Leaf	**3070**	444	486	**744**	129	101	30	**5004**	**6249**	261	312	109	–	**6931**	
Stem	433	470	**792**	502	–	84	92	2373	100	50	**1133**	434	–	1717	
	Conjugated amines (nmol. g^{-1} dw)														
	basic					neutral									
	Put	Spd	Spm	Dap	Cad	Put	Spd	HSpd	Spm	Dap	Cad	Phe	Tyr	M–Tyr	Octo	Dopa	3M, 4OH–Phe
Root	58	30	–	35	–	85	–	–	–	–	19	47	173	–	–	–	257
Leaf	172	–	–	95	–	289	558	93	–	–	–	–	**1343**	–	**1062**	–	502
Stem	72	–	–	56	67	–	–	–	–	–	–	–	89	–	–	–	541

Aromatic amines like tyramine appear to be characteristic of some tropical plants, especially monocots and some primitive families, like Cycadaceae (Ponchet, Martin-Tanguy, Marais, & Martin, 1982a). This is in good agreement with the systematical position of *Lyallia kerguelensis*. *Lyallia* is a monospecific genus, strictly endemic from Kerguelen Islands. Its very particular floral and vegetative characteristics, though placing it amongst Centrospermeae, excluded it from either the Portulacaceae or the Caryophyllaceae (Hennion, 1992). It was later linked with another endemic monospecific genus from New Zealand, *Hectorella* and the two were placed in the new family Hectorellaceae. A strong argument for the relictual character of *Lyallia* was presented with the finding of pollen from this species, together with pollen from the other endemic species *Pringlea antiscorbutica*, in late-glacial cores older than 10,000 BP, which suggested that these two species may have overwintered the Pleistocene epoch on Kerguelen (Young & Schofield, 1973). *Lyallia* and *Pringlea* were thus hypothesized as relicts of an otherwise extinct Tertiary Kerguelen flora.

Conjucated amine accumulation in the leaves of plants. Amides formed between hydroxycinnamic acids and amines are detected in high amounts in the flowering parts of various plants (Chiale, Cabrera, & Juliani, 1990; Sattar et al., 1990). Amides formed between hydroxycinnamic acids and amines are detected in high amounts in the flowering parts of various plants (reviewed in Martin-Tanguy (1985)). Other factors leading to an increase in conjugated amines in leaves are fungal and viral infections (reviewed in Martin-Tanguy (1985)). On the other hand, tolerance to several environmental challenges (mineral deficiencies, hypoxia, environmental pollutants, chilling) leads to the production of amine conjugates in leaves (reviewed in Bouchereau et al. (1999)). Thus, it was suggested that the protective effect of polyamines against the damage of superoxides was dependent on their prior conversion to conjugated forms. Furthermore, recent data support a role for conjugated tyramine as part of the array of defense chemicals and protective biopolymers induced in leaves and other plant tissues by wounding, to protect the plants against pathogen and herbivore attacks (Hohfeld, Schürmann, Schell, & Strack, 1995). The study of the distribution and content of free and conjugated polyamines and aromatic amines is thus a promising approach in view of the understanding of the physiological responses of subantarctic species to abiotic and biotic stresses. Our work is presently extending to other species analyzed in different sites or subjected to experimental stresses.

3. Experimental

3.1. Plant material

Whole plants were collected from the field at Kerguelen. Leaves, stems and roots were separated and fixed with liquid nitrogen, stored at −80°C then lyophilized. The dry material was used for extractions.

3.2. Analyses

3.2.1. Extraction, separation and quantification of free amines

Free amines were extracted in 1N HCl at a ratio of about 20 mg ml^{-1} HCl. After extraction for 1h in an ice bath, samples were pelleted at 48,000 × *g* for 20 min and the supernatant phase, containing the 'free' fraction, was stored frozen at −20°C. HPLC, TLC and fluorescence spectrophotometry were used to separate and quantify amines prepared as their dansyl derivatives. The method of Smith and Davies (1985) was used with some modifications as follows. Fifty to 100 μl aliquots of the supernatant were added to 200 μl of saturated sodium carbonate and 400 μl of dansyl chloride in acetone (7.5 mg ml^{-1}) in a 5 ml tapered reaction vial. After brief vortexing the mixture was incubated in darkness at room temperature overnight. Excess dansyl reagent was removed by reaction with 100 μl (100 mg ml^{-1}) of added proline and incubation for 30 min. Dansylated amines were extracted in 0.5 ml EtOAc. The organic phase was collected and stored in glass vials at −20°C. Standards were processed in the same way and 2–10 nmol were dansylated for each alone or in combination. In addition, the N_2-fixing eubacterium *Rhizobium*, which is known to contain high levels of homospermidine (Fujihara & Harada, 1989; Smith, 1977), was extracted and dansylated according to the methods described above.

3.2.2. HPLC

The column was a reverse phase ultrasphere C18 (particle size 5 μm; 4.6 mm × 250 mm, Berckman, Meroue, Galway, Ireland). Samples were eluted with a programmed methanol:water (v/v) solvent gradient, changing from 60 to 95% over 23 min at a flow rate of 1 ml min^{-1}, elution was completed after 7 min (Aziz, Martin-Tanguy, & Larher, 1997). For dansyl amines, an excitation wavelength of 365 nm was used with an emission wavelength of 510 nm. Hexanediamine was used as an internal standard. Confirmation of peak identities for amines was carried out by spiking the sample with known amounts of authentic standards. In addition, to identify homospermidine (Homospd) each sample was spiked with a dansylated crude extract of N_2-fixing eubacterium *Rhizobium*. The spermidine (Spd) standard curve was

used to quantify Homospd. Dansylamines were also separated and identified on precoated thin-layer silica gel (200–250 μm) such as silica gel G-60 (Merck). Samples were loaded onto a plate that had been activated at 110°C for 1 h. The plate was developed in a solvent system of cyclohexane:ethyl acetate (1:9 v/v), benzene:methanol (9:1 v/v) or chloroform:triethylamine (25:5 v/v) (Birecka, Di Nolfo, Martin, & Frohlich, 1984; Seiler, 1971). After chromatography, the dansyl derivatives were visualized under a UV light source (360 nm). Fluorescent spots were identified by comparing their R_f values with those of amine standards and Homospd found in the dansylated *Rhizobium* extract run on the same plate.

3.2.3. Extraction, separation and quantification of conjugated amines

The techniques which were used to extract, separate and identify the conjugated amines were as previously described (Cabanne, Dalebroux, Martin-Tanguy, & Martin, 1981; Martin-Tanguy, Cabanne, Perdrizet, & Martin, 1978; Ponchet et al., 1982a; Ponchet, Martin-Tanguy, Poupet, Marais, & Beck, 1982b). Plant tissue was extracted in a blender with 80% aqueous methanol at a ratio of about 40 mg ml^{-1}. After centrifugation ($10{,}000 \times g$ for 15 min) the supernatant was evaporated in vacuo at 30°C down to ca 10 ml, diluted with H_2O and then treated with: (1) petroleum ether that removes most of both chlorophylles, this fraction was discarded, (2) benzene that removes aromatic amine conjugates, (3) chloroform that removes neutral Put conjugates and (4) ethyl acetate that removes neutral Spd conjugates. The aqueous solution containing basic amides and some glucosides of aromatic amine conjugates was passed through a column of Amberlite resin Serva CG50 (H^+ form) and the column washed with H_2O and ethanol 40%. These eluates contained the glucosides of aromatic amine conjugates. Further elution with 3 M acetic acid provided basic conjugated polyamines. The eluate was taken to dryness and the residue dissolved in methanol and applied to a Polyclar AT (Serva) column. The column was eluted with methanol. Fractions were collected and analyzed. The elution pattern Polyclar AT in methanol followed the order: feruloyldiaminopropane, feruloylputrescine, feruloylspermidine and feruloylspermine. Methods used for the identification of basic and neutral conjugated amines included UV spectral data, fluorescence in UV light (254 and 366 nm alone and with NH_3), R_f values in 2D PC (Whatman No. 2) using BuOH–EtOH–H_2O (4:1:2) and H_2O, colour reaction with ninhydrin, identification of phenolic acid and the amine produced by hydrolysis (Ponchet et al., 1982a, b). Final confirmation of the structure was obtained by comparison with compounds (feruloylputrescine, feruloylspermidine, diferuloylputrescine, diferuloylspermidine, feruloyltyramine) synthesized by Roussel-Uclaf Industries (Paris). For the quantification of conjugated amines extracts from the different fractions (organic aqueous, ethanolic and acetic acid fractions concentrated to dryness) were boiled for 10–16 h in 6 N HCl, followed by measurements of free amines.

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