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PII: S0144-8617(96)00120-8

Carbohydrate Polymers **31** (1996) 1–9

0144-8617/96/$15.00 + .00

A survey and partial characterization of ice-nucleating fluids secreted by giant-rosette (*Lobelia* and *Dendrosenecio*) plants of the mountains of eastern Africa[1]

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(Received 30 April 1996; revised version received 9 August 1996; accepted 13 August 1996)

Ice-nucleating activities of the fluids from east African giant lobelias (13 samples from 10 taxa of *Lobelia*, Lobeliaceae) and giant senecios (4 samples from 4 taxa of *Dendrosenecio*, Asteraceae) were determined using supercooling temperatures as the indicator. Variation in supercooling temperatures of these fluids was found both within and between species. Fluids from *L. gregoriana* subsp. *gregoriana*, *L. deckenii* subsp. *deckenii*, *L. telekii* (Mount Kenya), *D. brassiciformis*, *L. gregoriana* subsp. *elgonensis*, *L. rhynchopetalum*, *D. battiscombei*, *D. keniensis*, and *D. cheranganiensis* subsp. *dalei* had the highest supercooling temperatures and were assumed to contain the most effective ice-nucleators. The first four of these were chosen for more detailed examination.

The most effective ice-nucleating fluids contained either a polysaccharide fraction composed primarily of uronic acid units or a pair of polysaccharide fractions containing little or no uronic acid. Polysaccharides isolated via 70% ethanol precipitation from fluids of *L. gregoriana* subsp. *gregoriana*, *L. deckenii* subsp. *deckenii*, and *D. brassiciformis* gave the two fractions on DEAE-cellulose chromatography. Main fractions were composed primarily of neutral sugars (glucose, mannose, fucose, rhamnose, and/or galactose); smaller amounts of galacturonic and glucuronic acids were also present. Fluid from *L. telekii* gave a single main fraction that chromatographed as an almost neutral polysaccharide although galacturonic acid was its principal sugar component. Supercooling temperatures of the isolated main fractions were lower than those of the original fluids except for that from *L. gregoriana* subsp. *gregoriana*, but all still exhibited ice-nucleating activities. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Giant lobelias (*Lobelia*, Lobeliaceae) and giant senecios (*Dendrosenecio*, Asteraceae) have diversified to occupy a variety of habitats on the isolated mountains of eastern Africa (Knox, 1993a; Fig. 1). There are 21 species of giant lobelia in eastern Africa, three of which have subspecies, for a total of 26 taxa (Knox, 1993b). They grow from Ethiopia to South Africa and from the coastal mountains of Tanzania inland to Zaire at altitudes ranging from 725–4400 m (Knox, 1993b). The 11 species of giant senecio, 5 of which have subspecies and varieties (for a total of 17 taxa), occupy a more restricted geographic and altitudinal range, growing in Zaire, Rwanda, Uganda, Kenya, and Tanzania at altitudes ranging from 2500–4600 m (Knox, 1993b). Habitats in which both groups are found include upper montane forest; mist-forest; high altitude, wet sedge-meadows; and the peculiar Afro-alpine zone. The climate in this zone was characterized as 'summer every day and winter every night' (Hedberg, 1964) because the diurnal temperature fluctuation greatly exceeds the annual variation in mean temperature, and nightly frost is common.

Giant lobelias and the giant senecios have independently evolved a giant-rosette growth-form, with numerous stunning examples of convergent evolution, both within and between the two groups. The fact that

[1]Journal paper No. 14903 of the Purdue Agricultural Experiment Station.
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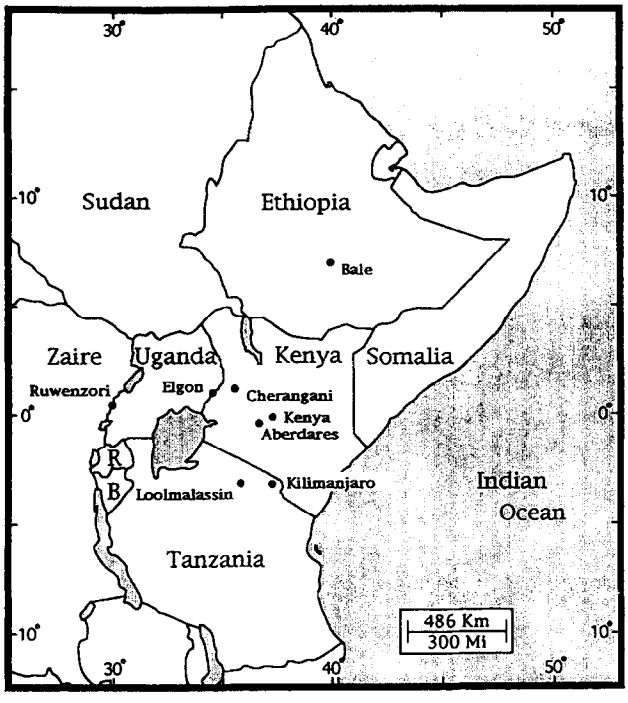

Fig. 1. Sampling sites of *Lobelia* and *Dendrosenecio* fluids (B—Burundi, R—Rwanda).

these unusual morphological features are present in *Lobelia* and *Dendrosenecio* species found growing side-by-side in various habitats provides strong *a priori* evidence that these features are adaptations to immediate environmental conditions. Convergent features of plants growing at higher altitudes almost certainly represent thermal adaptations to the diurnal fluctuations from ca. +20°C (daytime) to ca. −10°C (nighttime) (Beck, 1987). Putative adaptations include secretion and impoundment of fluids containing ice-nucleating polysaccharides (Beck *et al.*, 1982; Hedberg, 1964; Krog *et al.*, 1979).

The nature of polysaccharide secretion differs among taxa of giant lobelias and giant senecios. Most taxa have no obvious secretion of polysaccharides. Some taxa have developing leaves covered with a layer of mucilaginous fluid. It is not known whether this mucilaginous fluid serves as lubricant within the apical bud, or as a cryoprotectant for the young tissue of the developing leaves, or has some other function or a combination of functions. If leaf rosettes of these taxa are capable of impounding fluid, small quantities of fluid diluted with rainwater can be found among the leaf bases; it is not known if this fluid accumulates in the absence of rainwater. Other taxa secrete and impound large quantities of polysaccharide-containing fluids that cover the shoot apex and young leaves in the leaf rosette or, in the case of *L. telekii*, partially fills the hollow inflorescence.

Taxa with large quantities of impounded fluids in the leaf rosette have imbricate leaves. Rosettes can impound up to 1 liter of fluid in giant lobelia taxa and more than 3 liters of fluid in mature individuals of *D. brassicifolmis*. Rainwater mixes with these fluids, and although the critical experiments of fluid removal and covering to exclude rain have not been performed, it seems clear that the fluids are secreted because the rosettes are full during dry weather (Edwards & Taylor, 1935; Hedberg, 1964; Young & Van Orden Robe, 1986). The fluid is thought to be secreted by leaf bases (Hedberg, 1964; Coe, 1967). No significant secretory structures have been detected on the leaf surfaces, but fluid to be secreted was found in vacuoles of epidermal cells (Beck *et al.*, 1982). Secreted substance(s) was originally thought to lower the freezing point of the fluid (Coe, 1967), but it was later noted that no such freezing-point depression occurs (Young & Van Orden Robe, 1986; Beck et al., 1982). It was also suggested that its purpose was to reduce evaporation (Young & Van Orden Robe, 1986). Beck *et al.* (1982) reported that the substance secreted by *L. gregoriana* subsp. *gregoriana* (syn: *L. keniensis, L. deckenii* subsp. *keniensis*) was neither a pectic substance nor a typical exudate gum because no carboxyl groups were detected. However, Young & Van Orden Robe (1986) identified the substance as a pectin. In both investigations, the active compound(s) was neither isolated nor characterized.

The inflorescence of *L. telekii* is about 2 meters tall, with an inner diameter of 5–8 cm, and usually contains 1–2 liters of slightly viscous cryoprotecting fluid. This fluid has also been studied, but only cursorily, by Krog *et al.* (1979) who concluded that (a) the vital tissues of the plants are protected by the heat of fusion of water, (b) freezing of water is ensured by the presence of ice-nucleating agents, (c) the nucleating agents are carbohydrate in nature, and (d) the central fluid, which freezes, contains an unidentified polysaccharide.

This investigation was undertaken to characterize the ice-nucleating fluids and to evaluate whether patterns of variation are present that are congruent with DNA-based phylogenetic patterns (Knox and Palmer, unpublished data). Fluids were collected from all taxa of giant lobelia and giant senecio that had retrievable quantities of fluid among the leaf bases, impounded in the leaf rosette, or in the hollow inflorescence. A survey of 17 of these fluids and more detailed analyses of the ice-nucleating polysaccharides from *L. telekii*, *L. gregoriana* subsp. *gregoriana*, *L. deckenii* subsp. *deckenii*, and *D. brassicifolmis* are reported here. The results from additional studies on other species will be reported elsewhere.

EXPERIMENTAL

Materials

Seventeen samples of fluids were collected from taxa of *Lobelia* (13 samples) and *Dendrosenecio* (4 samples) during fieldwork conducted in eastern Africa in 1988 and 1989 (Table 1, Fig. 1). Fluids were removed from

Table 1. Sampling sites of *Lobelia* and *Dendrosenecio* fluids[a]

Plant sampled	Accession number	Site	Altitude (m)	Sample no. in Tables 2–4
Dendrosenecio battiscombei	Knox 750[b]	Mt Kenya	3450	6
D. brassiciformis	Knox 741	Aberdares	3925	5
D. cheranganiensis subsp. *dalei*	Knox 723[c]	Cherangani	3400	9
D. keniensis	Knox 766	Mt Kenya	3875	8
Lobelia aberdarica	Knox 700[b]	Mt Elgon	3300	11
L. bequaertii	Knox 206	Ruwenzori	3450	15
L. burttii subsp. *telmaticola*	Knox 782	Loolmalassin	3325	17
L. deckenii subsp. *deckenii*	Knox 823	Kilimanjaro	3950	4
L. gregoriana subsp. *elgonensis*	Knox 716	Cherangani	3525	1
L. gregoriana subsp. *elgonensis*	Knox 699	Mt Elgon	3700	14
L. gregoriana subsp. *gregoriana*	Knox 763	Mt Kenya	3800	2
L. gregoriana subsp. *sattimae*	Knox 740	Aberdares	3925	10
L. telekii	Knox 739	Aberdares	3925	16
L. telekii	Knox 689	Mt Elgon	3825	12
L. telekii	Knox 761	Mt Kenya	3925	7
L. rhynchopetalum	Knox 207	Bale	3850	3
L. wollastonii	Knox 205[b]	Ruwenzori	3850	13

[a]See also Fig. 1.
[b]Mixed sample from several nearby individuals with small quantities of fluid present.
[c]Mixed sample from several nearby individuals with very small quantities of fluid present.

the plants and transferred to plastic bottles using a bulb-style turkey baster or, for small quantities, the barrel of a 10-mL syringe. When possible, samples (up to 1 L) were collected from a single plant; but for plants producing small quantities of fluid, fluids collected from several nearby plants were pooled. Samples were kept as cool as possible during transport down mountains (1–7 d), stored in a freezer prior to transport to the US (which involved thawing for less than 24 h), and then refrozen and kept frozen prior to analysis. Analyses were conducted on both intact and centrifuged fluids.

General

Total protein was determined using the protein assay kit of the Sigma Chemical Co. (St Louis, MO) employing bicinchoninic acid (Smith *et al.*, 1985) and BSA as the standard. Total carbohydrate was determined by the phenol–sulfuric acid method (Dubois *et al.*, 1956) using glucose as the standard. Percent total solids was determined by drying samples in a vacuum oven at 50°C to constant weight. Viscosities of intact fluids were determined at 25°C using a Brookfield digital viscometer (Model LVT with a small sample adapter; Brookfield Engineering Labs, Stoughton, MA).

Ice-nucleating activity

The supercooling temperature (the lowest temperature reached by the solution on cooling prior to the sudden increase in temperature due to release of the latent heat of fusion when the water crystallized) was used as the indicator of ice-nucleating activity (Krog *et al.*, 1979; Blond, 1985). Supercooling temperatures (ST) were determined using 2 mL of fluid in a 13×100-mm screw-capped Pyrex test tube with a 24-gauge copper-constantan thermocouple placed at the center of the fluid. The tube was immersed in a 50% ethylene glycol bath, and the temperature was lowered from 15°C to −15°C at a rate of 0.20°C/min. Temperatures were recorded every 10 s with a Kaye digistrip II data logger (Kaye Instruments, Bedford, MA). ST of both intact (sample as received) and centrifuged (14 600 × g, 15 min) plant fluids were determined. The residues from centrifugation were reconstituted to the original concentration to determine the ice-nucleating effectiveness of the particulate (insoluble) material. The ST of 0.2% solutions of the 70% ethanol precipitate from the supernatant and 0.1% solutions of the main fraction after DEAE-cellulose chromatography of the ethanol precipitate were also determined.

Fractionation of the isolated polysaccharides on DEAE-cellulose

A solution (0.25 to 1.00%) of the ethanol precipitate from the supernatant of the plant fluid was fractionated on a 1.0×20-cm column of DEAE-cellulose (DE 51, Whatman Biosystems Ltd, Kent, England). NaCl gradients (0–0.10 M, 0.08–0.30 M, 0.25–0.50 M) were used in sequence to elute the components. Each fraction (8.0 mL) was analyzed for total carbohydrate using the phenol–sulfuric method (Dubois *et al.*, 1956) and for uronic acid content using the *m*-hydroxydiphenyl method (Filisetti & Carpita, 1991).

Monosaccharide composition

Monosaccharide components of the main fractions from DEAE-cellulose chromatography were determined

after hydrolysis (Yadav *et al.*, 1994) using a Dionex BioLC high-performance liquid chromatography system with a Carbopac PA1 column, a PAD-2 pulsed amperometric detector, and a Spectra-Physics 4400 integrator (Sunnyvale, CA). The eluants were water for the first 35 min and 100 mM NaOH + 150 mM NaOAC for the next 35 min; 400 mM NaOH was added post-column to the eluant.

RESULTS AND DISCUSSION

Survey of ice-nucleating activities of *Lobelia* and *Dendrosenecio* fluids

Results for 17 representative samples are given in Tables 2–4. In the tables, fluids are grouped according to their effectiveness as ice-nucleating agents, with group 1 (Table 2) being the most effective for both the original and centrifuged samples and group 3 (Table 4) being the least effective. Deionized, glass-distilled, membrane-filtered, and degassed water, which was used to make all solutions for ST determination, froze at -11.6 ± 0.5°C. A 1% solution of carboxymethylcellulose, an ice cream stabilizer that keeps ice crystals small, froze at -9.0 ± 0.6°C under the same conditions.

Fluid from *L. gregoriana* subsp. *elgonensis* (Cherangani Hills) had the highest ST (−3.5°C). Fluids from *L. gregoriana* subsp. *gregoriana*, *L. rhynchopetalum*, *L. deckenii* subsp. *deckenii*, *D. brassiciformis*, *D. battiscombei*, *L. telekii* (Mt Kenya), *D. keniensis*, and *D. cheranganiensis* subsp. *dalei* also had high freezing temperatures. It was assumed that these nine fluids contained the most effective ice nucleators. *L. telekii* fluids (intact and centrifuged) froze at ca. −5.0°C. Variation in ST of fluids was noted both within and between species.

The ability of some *Lobelia* and *Dendrosenecio* fluids to freeze at higher temperatures than 'pure' water was not correlated with their total carbohydrate, total protein, or total solids contents or viscosities (Tables 2–4). Fluids from *L. rhynchopetalum* had more than ten times the content of total carbohydrate and total protein than fluids from *L. gregoriana* subsp. *elgonensis* (Cherangani Hills), but supercooling temperatures of their intact and centrifuged fluids were almost the same. Fluid from *D. keniensis* had the lowest % total solids (0.12%); yet its ST was higher than those of fluids from *L. aberdarica*, *L. telekii* (Aberdares and Mt Elgon), and *L. burtii* subsp. *telmaticola*, which had much higher total solids contents. The sample with the highest viscosity was the fluid from *D. cheranganiensis* subsp. *dalei* (53 mPa s×sec). Its ST was high, but not much higher than that of the low-viscosity fluids of *L. gregoriana* subsp. *elgonensis* (Cherangani Hills), *L. rhynchopetalum*, *D. brassiciformis*, and *L. telekii* (Mt Kenya). Although the viscosity of *L. telekii* fluid was very low, its suspending properties prevented the insoluble particles from settling.

Three *Lobelia* species and one *Dendrosenecio* species were selected for more detailed study (Table 5). Fluid collected from *L. telekii* was that produced and stored inside the inflorescence (Fig. 2), unlike fluids of the dendrosenecios and other lobelias, which are secreted from and entrapped by the leaves of the rosette.

Supercooling temperatures of solutions of isolated components reconstituted to their original concentrations are given in Table 5. There were no significant differences between the ST of soluble and particulate fractions obtained by centrifugation, except for *L. deckenii* subsp. *deckenii*. Supercooling temperatures of solutions of the EtOH precipitates of all samples were slightly lower than those of intact fluids, except for that of *L. gregoriana* subsp. *gregoriana*, which remained the same. None of the 70% EtOH solubles had ice-nucleating activities comparable to those of the intact fluids. Supercooling temperatures of the main fractions of the EtOH precipitates (0.1% solutions) likewise were lower than those of both intact fluids and EtOH precipitates. However, intact fluids, sediments (reconstituted to the same concentration as the intact fluid), ethanol precipitates (0.2% solution), and main fractions (0.1% solutions) of the selected species all exhibited ice-nucleating activities. The lowest ST recorded were −6.9° and −7.0°C for 0.1% solutions of main fractions of *L. deckenii* subsp. *deckenii* and *L. telekii*, respectively. The reason for this decrease has yet to be determined and might be due to differences in concentration and/or fewer molecular aggregates. Each insoluble residue examined had much the same monosaccharide composition as its respective soluble polysaccharide(s) (data not given) and about the same ST as the intact fluid from which it was obtained (Table 5), which suggests that aggregation may play a role in ice nucleation. It is also likely that insoluble particles may have been formed by freezing and thawing. Repeated freezing and thawing of the *L. telekii* centrifugate in the laboratory produced insoluble particles.

Polysaccharide components via DEAE-cellulose chromatography

The EtOH precipitate from the supernatant of the centrifuged fluid of *L. telekii* contained one major fraction (Fig. 2) of high galacturonic acid content, which suggests that it is a pectic substance.

Ethanol precipitates from supernatants of centrifuged fluids of *D. brassiciformis*, *L. gregoriana* subsp. *gregoriana*, and *L. deckenii* subsp. *deckenii* each contained two major fractions (Fig. 2), one fraction contained only small amounts of uronic acid. The main fraction of 70% ethanol-precipitated polysaccharide from *L. telekii* eluted at the same position as the polysaccharides of the

Table 2. GROUP 1 – Lobelia and Dendrosenecio fluids with supercooling temperatures (ST) between −3.5° and −6.1° for both the original and centrifuged samples

No.	Plant	Fluid description	Supercooling temp.[c] (°C)		Total carbohydrate[d] (mg/mL)		Total protein[e] (mg/mL)		Total solids (%)	Viscosity[f] (mPa s)
			Intact[a]	Centrifuged[b]	Intact[a]	Centrifuged[b]	Intact[a]	Centrifuged[b]	Intact[a]	Intact[a]
1.	*Lobelia gregoriana* subsp. *elgonensis*	light brown, viscous, suspended particles and short fibers	−3.5±0.1	−4.3±0.2	0.436±0.039	0.422±0.024	0.135±0.016	0.090±0.003	1.0011	7
2.	*L. gregoriana* subsp. *gregoriana*	clear, slightly viscous	−4.0±0.1	−4.2±0.1	0.178±0.011	0.112±0.004	0.046±0.003	0.020±0.001	0.9911	6
3.	*L. rhynchopetalum*	light brown, viscous, many suspended particles	−4.5±0.1	−4.3±0.2	6.681±0.275	6.926±0.500	4.157±0.274	5.296±0.203	0.69	10
4.	*L. deckenii* subsp. *deckenii*	clear with white suspended particles	−4.5±0.4	−6.0±0.9	0.153±0.008	0.121±0.005	0.050±0.002	0.031±0.001	0.9829	3
5.	*Dendrosenecio-brassiciformis*	many light brown particles	−4.8±0.3	−5.5±0.5	0.346±0.015	0.304±0.022	0.140±0.022	0.064±0.003	1.0106	3
6.	*D. battiscombei*	light brown, viscous fluid; suspended material; slightly sweet odor	−4.8±0.1	−5.5±0.1	1.660±0.110	1.382±0.031	0.282±0.006	0.320±0.017	0.38	g
7.	*L. telekii*	slightly viscous, suspended particles	−5.3±0.5	−4.9±0.4	0.745±0.081	0.653±0.086	0.978±0.048	0.974±0.030	0.32	2
8.	*D. keniensis*	light brown fluid, many suspended particles, sweet odor	−5.6±0.3	−6.1±0.5	0.529±0.015	0.452±0.024	0.280±0.000	0.335±0.009	0.12	7
9.	*D. cheranganiensis* subsp. *dalei*	light brown fluid; many suspended particles, most fibrous; sweet odor	−5.7±0.4	g	1.196±0.007	g	1.670±0.048	g	0.21	53

[a]As received.
[b]After centrifugation at 14 600× g for 15 min.
[c]The supercooling temperature was determined using a 24-gauge copper-constantan thermocouple immersed in a 2.0-mL sample in an acid-washed Pyrex culture tube (13×100 mm) closed with a screw cap. Tubes were immersed in a MGW Lauda Brinkman (RM 6) bath with a slowly decreasing temperature. Each value is the mean value of at least 8 tubes.

For reference purposes, the ST of water was determined using water purified through a Barnstead Nanopure II purifier, membrane filtered (0.45 μm), and degassed by boiling and storing in a flask equipped with a soda lime-filled tube. The pH was 6.96 and the S.T. −11.64±0.60°C.
[d]Total carbohydrate was determined by the phenol-sulfuric acid method using D-glucose as the standard. Analyses were done in triplicate.
[e]Total protein was determined using the bicinchoninic acid protein assay (Sigma Chemical Co.) and BSA as the standard. Analyses were done in triplicate.
[f]Total solids were determined using a vacuum oven set at 50°C in duplicate. Viscosity was determined using a Brookfield digital viscometer (Model LVT). Viscosity of distilled water under the same conditions was 2 cps.
[g]Analyses not done because of small sample size.

Table 3. GROUP 2 - *Lobelia* and *Dendrosenecio* fluids with ST between −3.5° and −6.1° for the original fluid but with ST < −6.1° for the centrifuged sample

No.	Plant	Fluid description	Supercooling temp.[c] (°C)		Total carbohydrate[d] (mg/mL)		Total protein[e] (mg/mL)		Total[f] solids (%)	Viscosity[f] (mPa s)
			Intact[a]	Centrifuged[b]	Intact[a]	Centrifuged[b]	Intact[a]	Centrifuged[b]	Intact[a]	Intact[a]
10.	*L. gregoriana* subsp. *sattimae*	brown fluid, suspended particles	−4.8±0.1	−7.8±0.8	0.461±0.039	0.196±0.006	0.086±0.003	0.042±0.002		8
11.	*L. aberdarica*	light brown fluid, large masses of fibrous material, sweet odor	−4.9±0.4	−7.8±0.4	1.980±0.062	2.060±0.098	0.961±0.021	1.034±0.051	0.9862	4
12.	*L. telekii*	turbid fluid, large suspended particles, slightly sweet odor	−5.2±0.2	−7.3±0.4	6.387±0.396	6.287±0.529	3.808±0.094	4.595±0.200	0.9457	3

[a–f]See Table 2.

Table 4. GROUP 3 - *Lobelia* and *dendrosenecio* fluids with ST below −6.1°

No.	Plant	Fluid description	Supercooling temp.[c] (°C)		Total carbohydrate[d] (mg/mL)		Total protein[e] (mg/mL)		Total[f] solids (%)	Viscosity[f] (mPa s)
			Intact[a]	Centrifuged[b]	Intact[a]	Centrifuged[b]	Intact[a]	Centrifuged[b]	Intact[a]	Intact[a]
13.	*L. wollastonii*	turbid, suspended particles, sweet medicinal odor	−6.2±0.02	−7.8±0.3	0.280±0.004	0.195±0.004	0.513±0.016	0.480±0.008		1
14.	*L. gregoriana* subsp. *elgonensis*	light brown, turbid large suspended particles	−6.4±0.4	−7.0±0.8	0.276±0.032	0.182±0.001	0.098±0.002	0.094±0.005		g
15.	*L. bequaertii*	clear, slightly viscous, white suspended particles	−6.6±0.3	−7.2±1.0	0.098±0.001	0.067±0.001	0.028±0.002	0.025±0.001		3
16.	*L. telekii*	clear	−7.7±1.3	−8.1±1.3	4.811±0.168	4.895±0.315	1.854±0.003	1.874±0.059	1.0029	1
17.	*L. burttii* subsp. *telmaticola*	slightly turbid, suspended fibrous materials, slightly foul odor	−8.5±1.3	−9.0±1.1	0.067±0.004	0.046±0.000	0.027±0.004	0.015±0.001	0.9665	2

[a–g]See Table 2.

Table 5. Supercooling temperatures of fluids from selected *Lobelia* and *Dendrosenecio* plants and solutions of fractions

Sample	Supercooling temp. (°C)	Total carbohydrates[d] (mg/mL)	Total proteins[d] (mg/mL)
Lobelia telekii (Mt. Kenya)			
Intact[a]	−5.3±0.5	0.745±0.081	0.978±0.048
Soluble[b]	−4.9±0.4	0.653±0.086	0.974±0.030
Sediment[b,e]	−5.2±0.8	0.172±0.021	0.172±0.021
EtOH solubles[e]	ND[f]	ND	ND
EtOH precipitate[g]	−6.0±0.4	0.655±0.072	0.098±0.002
Main fraction[h]	−7.0±0.4	0.397±0.024	0.076±0.000
Dendrosenecio brassiciformis			
Intact[a]	−4.8±0.3	0.346±0.015	0.140±0.022
Soluble[b]	−5.5±0.5	0.304±0.022	0.064±0.003
Sediment[b,e]	ND	ND	ND
EtOH solubles[e]	ND	ND	ND
EtOH precipitate[g]	−5.8±0.1	ND	ND
Main fraction[h]	−6.0±0.6	ND	ND
L. gregoriana subsp. *gregoriana*			
Intact[a]	−4.0±0.1	0.178±0.011	0.046±0.003
Soluble[b]	−4.2±0.1	0.112±0.004	0.020±0.003
Sediment[b,e]	−4.0±0.3	0.077±0.002	0.027±0.003
EtOH solubles[e]	−7.9±0.4	0.091±0.003	0.048±0.004
EtOH precipitate[g]	−3.8±0.3	1.092±0.088	0.152±0.002
Main fraction[h]	−4.8±0.5	0.410±0.035	0.141±0.005
L. deckenii subsp. *deckenii*			
Intact[a]	−4.5±0.4	0.153±0.008	0.050±0.002
Soluble[b]	−6.0±0.9	0.121±0.005	0.031±0.001
Sediment[b,e]	−5.9±0.6	0.043±0.000	0.027±0.001
EtOH solubles[e]	−9.6±0.7	0.029±0.001	0.027±0.001
EtOH precipitate[g]	−5.9±0.2	1.636±0.104	0.121±0.004
Main fraction[h]	−6.9±0.3	0.591±0.074	0.201±0.004

[a,b,d,e] See Table 2.
[e]Reconstituted to original concentration.
[f]ND — not determined.
[g] At 0.2% concentration.
[h]Main fraction (fraction 2) of the EtOH precipitate from DEAE-cellulose chromatography at 0.1% concentration.

three other species, although it had a high content of uronic acid. This behavior might be due to a high degree of esterification causing the polysaccharide to chromatograph like an almost neutral polysaccharide.

Monosaccharide composition

The main fraction of 70% ethanol-precipitated polysaccharide from *L. telekii* contained 77 mole% galacturonic acid, 8 mole% arabinose, and about 12 mole% of additional neutral sugars (Table 6). It had a 3 mole% glucuronic acid content, so the total uronic acid content was 80 mole%. This polysaccharide had a monosaccharide composition profile similar to that of a native pectin. The number and percentage of individual component monosaccharides, chemical structures, and molecular weights of pectins vary with the source, the conditions employed for extraction, and any other treatments used in their preparation (BeMiller, 1986); but most pectins contain ca. 80% D-galacturonic acid, 10–20% total neutral sugars, and ca. 2% L-rhamnose (Van Buren, 1991). Neutral sugars other than L-rhamnose commonly found in pectins are D-galactose, L-arabinose, and D-xylose and less frequently, D-glucose, D-mannose, and L-fucose (Van Buren, 1991). Small amounts of D-glucuronic acid are found in some pectins (Van Buren, 1991). The principal neutral sugars in the main fraction of *L. telekii* fluid were arabinose and rhamnose. L-Arabinose was the primary neutral sugar present in pectins extracted from apple, sugar beet (Renard & Thibault, 1993), and Siberian apricot (Odonmazig *et al.*, 1992). Pectins from Siberian apricot also contained a high amount of L-rhamnose (Odonmazig *et al.*, 1992). The main fraction from *L. telekii* fluid also contained glucose, xylose, galactose, and fucose in smaller amounts.

The main fraction (fraction 2, Fig. 2) from *D. brassiciformis* fluid contained less than 1 mole% uronic acid.

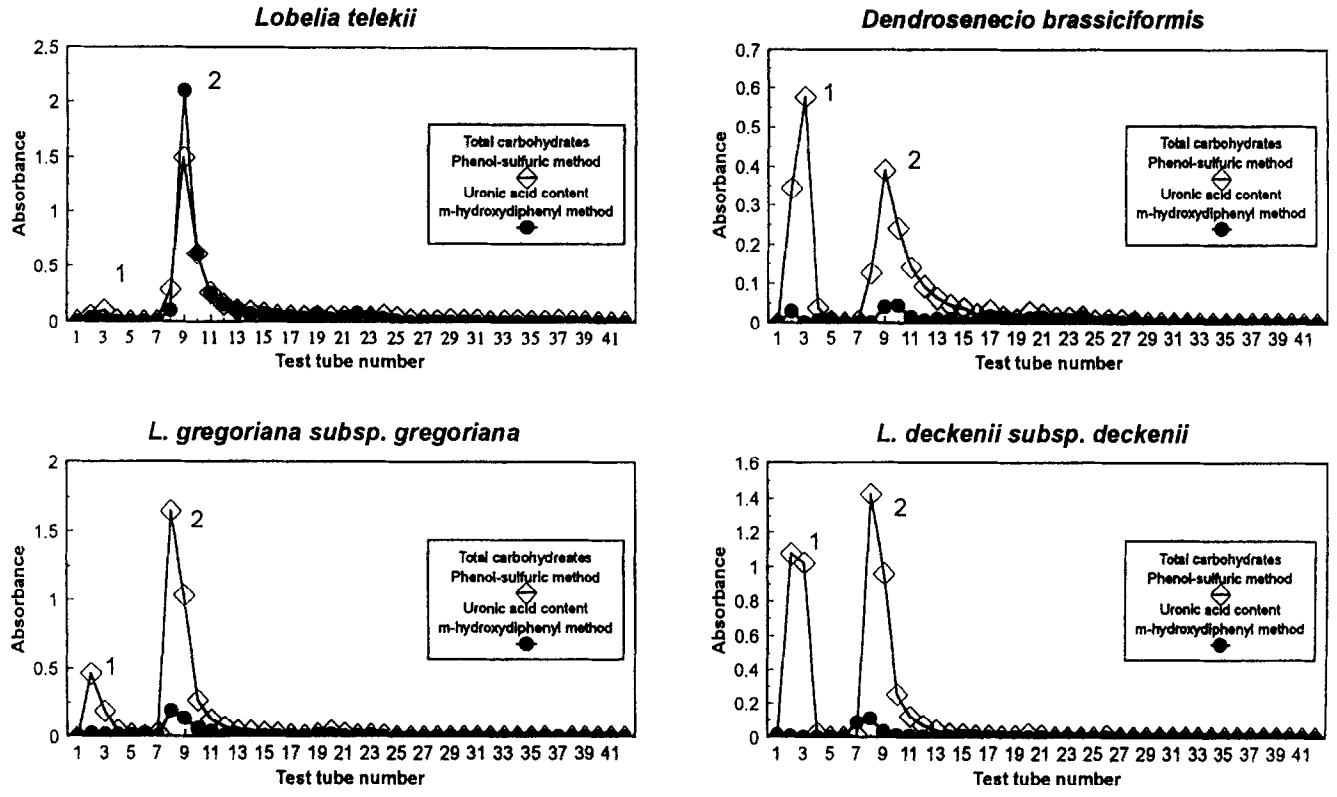

Fig. 2. Elution patterns of ethanol precipitated polysaccharides from selected *Lobelia* and *Dendrosenecio* species. The following eluants were used: Tubes #0–7, water; #8–19, 0 to 0.10 M NaCl; #20–31, 0.08–to 0.30 M NaCl and #32–43, 0.25–0.50 M NaCl.

Table 6. Sugar composition (mole%) of the main fraction (fraction 2) of *Lobelia* and *Dendrosenecio* fluids

Sample	Fucose	Rhamnose	Arabinose	Galactose	Glucose	Xylose	Mannose	Galacturonic acid	Glucuronic acid
D. brassiciformis	19.28±1.78	16.04±0.10	0	15.65±2.11	27.45±0.91	0	20.85±0.35	0.19±0.15	0.54±0.17
L. gregoriana subsp. *gregoriana*	24.36±2.00	3.74±0.60	4.66±0.32	15.26±2.11	24.88±2.50	0	13.43±0.12	7.35±0.18	6.32±0.15
L. deckenii subsp. *deckenii*	14.88±0.84	10.34±1.03	2.58±0.20	22.24±1.15	24.16±1.25	0	12.47±0.76	8.75±0.45	4.58±0.25
L. telekii	0.92±0.04	5.55±0.85	8.04±0.84	0.92±0.62	2.62±0.82	2.13±0.46	0	76.73±2.76	3.08±0.79

Its main sugar was glucose (ca. 27 mole%), followed by mannose and fucose at almost the same concentration (ca. 20 mole%) and rhamnose and galactose at ca. 16 mole%.

Main fractions (fraction 2, Fig. 2) from fluids of *L. gregoriana* subsp. *gregoriana* and *L. deckenii* subsp. *deckenii* had very similar sugar composition profiles. Both contained about 13 mole% uronic acid. The main neutral sugar of the two samples was glucose (ca. 24 mole%). Although both samples contained galactose and fucose, and the total mole% of these two related sugars was about the same, their relative concentrations were reversed; the main fraction of *L. deckenii* subsp. *deckenii* had more rhamnose than did the main fraction of *L. gregoriana* subsp. *gregoriana*. The mannose contents of both samples were almost the same (ca. 13 mole%). The ice-nucleating polysaccharides of these two related species had similar monosaccharide profiles.

Evolutionary interpretation

Impounded ice-nucleating fluids in *Lobelia* and *Dendrosenecio* had similarities, but the variation in the results, particularly among samples from isolated populations or subspecies in a species, precluded extensive interpretation. Although highest ST were found in *Lobelia* species, ST of samples from *Dendrosenecio* were in the middle of the overall range (Tables 2–4). The sugar composition in the sample of *D. brassiciformis*

was distinguished from the three *Lobelia* samples by lack of arabinose and the small proportions of D-galacturonic and D-glucuronic acids, but there were more differences in sugar composition between the inflorescence-impounded fluid of *L. telekii* and the three rosette-impounded fluids than there were between the rosette-impounded fluid of *D. brassiciformis* and the two rosette-impounded fluids from *Lobelia*. The general similarity in properties and composition between *L. gregoriana* subsp. *gregoriana* and *L. deckenii* subsp. *deckenii* may be due to their close relationship in a clade that also includes *L. burttii* (the Deckenii Group), but the next most inclusive clade also includes *L. telekii* and *L. aberdarica* (Knox and Palmer, unpublished data). The very distinctive sugar composition of *L. telekii* indicates that the evolution of its inflorescence-impounded fluid (which is only found in this species) involved more than a simple transfer of secretory activity from the rosette to the inflorescence, and the possibility remains open that the inflorescence-impounded fluid of *L. telekii* evolved independently of the rosette-impounded fluids. Variation seen in fluid properties among populations of *L. telekii*, subspecies of *L. gregoriana*, and species of the Deckenii Group indicates that much more work is required to assess phylogenetic, developmental, and environmental sources of variation.

ACKNOWLEDGEMENTS

EBK thanks the Tanzanian Commission for Science and Technology, the Ugandan National Research Council, the National Museums of Kenya, and the Addis Ebeba University and the Ethiopian Ministry of Foreign Affairs for permission to conduct research in their respective countries. Field work was supported in part by grants from the National Science Foundation (BSR-8800487 and BSR-8901123).

REFERENCES

Beck, E., Senser, M., Scheibe, R., Steiger, H.-M. & Pongrats, P. (1982). Frost avoidance and freezing tolerance in afroalpine 'giant rosette' plants. *Plant, Cell Environ.*, **5**, 215–222.

Beck, E., Schulze, E.-D., Senser, M. & Scheibe, R. (1984). Equilibrium freezing of leaf water and extracellular ice formation in afroalpine 'giant rosette' plants. *Planta*, **162**, 276–282.

Beck, E. (1987). Mechanisms of freezing avoidance and freezing tolerance in tropical alpine plants. In *Plant Cold Hardiness*, ed. P.H. Li. Alan R. Liss, Inc. pp. 155–168.

BeMiller, J.N. (1986). An introduction to pectins: structure and properties. In *Chemistry and Function of Pectins*, eds M.L. Fishman & J.J. Jen. American Chemical Society, Washington, D.C., pp. 2–12.

Blond, G. (1985). Freezing in polymer–water systems and properties of water. In *Properties of Water in Foods*, eds D. Simatos & J.L. Multon. Martinus Nijhoff Publishers, Dordrecht, The Netherlands, pp. 531–542.

Coe, M.J. (1967). *The Ecology of the Alpine Zone of Mount Kenya*. Monographiae Biologicae, No. 17. Junk, The Hague.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Anal. Chem.*, **28**, 350–356.

Edwards, F.W. & Taylor, G. (1935). British Museum Expedition to East Africa. I. *Nat. Hist. Mag. (British Museum)*, **5**, 165–186.

Filisetti, T.M.C.C. & Carpita, N.C. (1991). Measurement of uronic acids without interference from neutral sugars. *Anal. Biochem.*, **197**, 157–162.

Hedberg, O. (1964). Features of afroalpine plant ecology. *Acta Phyt. Suec.*, **49**, 1–144.

Knox, E.B. (1993). The conservation status of the giant senecios and giant lobelias in eastern Africa. *Opera Bot.*, **121**, 195–216.

Knox, E.B. (1993). The species of giant senecio (Compositae) and giant lobelias (Lobeliaceae) in eastern Africa. *Contr. Univ. Mich. Herb.*, **19**, 241–257.

Knox, E.B., Downie, S.R. & Palmer, J.D. (1993). Chloroplast genome rearrangements and the evolution of giant lobelias from herbaceous ancestors. *Molec. Biol. Evol.*, **10**, 414–430.

Knox, E.B. & Palmer, J.D. (1995). Chloroplast DNA variation and the recent radiation of the giant senecios (Asteraceae) on the tall mountains of eastern Africa. *Proc. Natl. Acad. Sci., USA*, **92**, 10349–103563.

Krog, J.O., Zachariassen, K.E., Larson, B. & Smidsrød, O. (1979). Thermal buffering in Afro-alpine plants due to nucleating agent-induced water freezing. *Nature*, **282**, 300–301.

Odonmazig, P., Badga, D., Ebringerova, A. & Alfoldi, J. (1992). Structures of pectic polysaccharides isolated from Siberian apricot (*Armeniaca siberia* Lam.). *Carbohydr. Res.*, **226**, 353–358.

Renard, C.M.G.C. & Thibault, J.F. (1993). Structure and properties of apple and sugar-beet pectins extracted by chelating agents. *Carbohydr. Res.*, **244**, 99–114.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. & Klenk, D. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.*, **150**, 76–85.

Van Buren, J.P. (1991). Function of pectin in plant tissue structure and firmness. In *The Chemistry and Technology of Pectin*, ed. R.H. Walter. Academic Press, Inc., San Diego, CA, pp. 1–22.

Yadav, M.P., BeMiller, J.N. & Embuscado, M.E. (1994). Compositional analysis of polysaccharides via solvolysis with liquid hydrogen fluoride. *Carbohydr. Polym.*, **25**, 315–318.

Young, T.P. & Van Orden Robe, S. (1986). Microenvironmental role of a secreted aqueous solution in the Afroalpine plant *Lobelia keniensis*. *Biotropica*, **18**, 267–269.