PROTEIN ELECTROPHORETIC PATTERNS AND ANTI-FREEZING ACTIVITY IN THE LEAF APOPLAST OF THE TROPICAL ANDEAN SPECIES *Senecio niveoaureus*

Patrones electroforéticos de proteínas y actividad anticongelante en el apoplasto de la hoja de la especie andina tropical *Senecio niveoaureus*

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ABSTRACT

Tropical high mountain plants have different adaptations to survive extreme daily temperature fluctuations and specially freezing night conditions. In winter plant species, survival to low temperatures is related to the ability of the cell to produce specific low molecular weight proteins (anti-freezing proteins) and to export them to the apoplast. In order to see if high mountain tropical plants survive to low temperatures through the same mechanism we collected, during a 24 hour-period, leaves from *Senecio niveoaureus* growing at 3,300 and 3,600 m.o.s.l, in the Páramo de Palacio, Chingaza, Colombia. Leaf apoplast proteins had MW between 35-12 kDa. Electrophoretic patterns were different depending on the altitude and the time of sampling. However the observed variations could not be linked to changes in temperature or to the altitudinal gradient. Antifreeze activity was detected in leaf apoplast of plants at different altitudes. This is the first report of anti-freeze activity in a high mountain tropical species.

Key words: anti-freezing proteins, *Senecio niveoaureus,* high tropical Andes (páramo).

RESUMEN

Las plantas de alta montaña tienen diferentes adaptaciones para sobrevivir a cambios drásticos de temperatura, especialmente a condiciones de congelamiento. En plantas de invierno, la supervivencia a temperaturas bajas está relacionada con la capacidad de las células para producir proteínas específicas de bajo peso molecular (proteínas anticongelantes) y exportarlas al apoplasto. Para establecer si plantas tropicales de alta montaña sobreviven las temperaturas bajas a través del mismo mecanismo, se colectaron hojas de plantas de *Senecio niveoaureus* durante 24 horas y a dos alturas 3.300 y 3.600 msnm en el Páramo de Palacio, Chingaza, Colombia. Se observaron

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proteínas apoplásticas de pesos moleculares entre 35-12 kDa. Los patrones electroforéticos fueron diferentes dependiendo de la altura y la hora de muestreo, sin embargo, se observaron variaciones en el patrón de bandeo que no pueden ser atribuidas ni a la temperatura ni al gradiente altitudinal únicamente. Se detectó actividad anticongelante en el apoplasto de hojas de *S. niveoaureus,* siendo este el primer reporte en especies tropicales de alta montaña.

Palabras clave: proteinas anticongelantes, *Senecio niveoaureus,* páramo.

Abbreviations: antifreeze proteins (AFP)

INTRODUCTION

Tropical high mountain regions present drastic changes in daily temperature, exposing plants to extreme conditions in which they must develop morphological, physiological and biochemical characteristics that allow them to survive. Tropical high mountain ecosystems, located at the upper limit of forests (2,800 m.o.s.l) and below the level of perpetual snow (4,200 m.o.s.l) are known as *páramos,* a term restricted to the Andes and mountains in the south of Central-America (Luteyn, 1999). Due to their location, *páramos* are characterized by wide daily thermal alternation where temperatures oscillate between -3 °C and 12 °C. The annual rainfall in *páramos* varies between 500 mm and 3,000 mm and there is intense ultraviolet radiation and strong winds (Mora-Osejo and Sturm, 1994; Lüttge, 1997; Luteyn, 1999). These ecological conditions of the *páramos* allow limited growth of specific plants highly adapted to the extreme conditions. Several protection mechanisms against low temperatures have been identified in *páramo* plants including morphological aspects such as rossetted habitat, the presence of necromass (Beck *et al.,* 1982; Rada *et al.,* 1985; Körner, 1999), coriacity, leaf orientation (Goldstein *et al.,* 1989), increased sugar content, mucilage secretion, reduced leaf bud freezing point (Beck *et al.,* 1982; Körner, 1999; Lüttge, 1997), increased lineal super-cooling ability as altitude increases and avoiding or resistance to low temperatures (Rada *et al.,* 1985; Rada *et al.,* 1987; Rada *et al.,* 2001). However, few biochemical studies have been done on *páramo* species and up to date there are no reports of anti-freeze protein activity.

The presence of anti-freeze proteins (AFP) is one of the mechanisms of plants to tolerate low temperatures. AFPs have been found in wheat, barley, rye, alfalfa (Hon *et al.,* 1994; Antikainen *et al.,* 1996; Antikainen and Griffith, 1997) and carrots (Worrall *et al.,* 1998; Smallwood *et al.,* 1999). Plant AFPs present partial homology with AFPs described in Atlantic flounder. AFPs have two related effects on aqueous solution: thermal hysteresis (TH) and inhibition of ice re-crystallization. TH is the non-colligative depression of the freezing temperature of solutions below their melting temperature. Ice re-crystallization is the growth of large ice crystals at the expense of the smaller ones in partially frozen solutions (Smallwood *et al.,* 1999). AFPs inhibit the growth and re-crystallization of intercellular ice by adsorbing it onto the surface of ice crystals via van der Waals interactions and/or hydrogen bonds (De Vries, 1986). In plants, AFPs are present in epidermal cell walls, in cell walls surrounding intercellular

spaces and in secondary cell walls of leaf xylem (Antikainen *et al.*, 1996; Pihakaski-Maunsbach *et al.*, 1996). Different studies have shown that some of the apoplast AFPs in rye have also glucanase and chitinase activities, which indicates a bi-functionality of these proteins acting as defense proteins against pathogens and against ice formation (Hon *et al.*, 1995; Griffith *et al.*, 1997). However, since glucanases and chitinases normally do not have anti-freeze activity, it has been hypothesized that AFPs evolved from the pathogenesis related proteins (Griffith *et al.*, 1997).

Senecio niveoaureus is a rossete plant from the Colombian *páramos*, found between 3,200 m.o.s.l and 4,300 m.o.s.l. In this species the mechanisms of adaptation to low temperatures are not well known, however, research with giant *Senecio* species from African alpine ecosystems has shown that leaves close to the apical bud move towards it keeping the temperature in the bud some degrees higher than the air. Also, the freezing point in apical bud cells is lower than in mature leaves. Furthermore, the presence of cryoprotectans such as sugars has been reported (Beck *et al.*, 1982; Körner, 1999). This study analyzed the electrophoretic patterns and the anti-freeze activity of proteins from the leaf apoplast of *S. niveoaureus* and their relationship with the altitudinal gradient and daily temperature changes in a day-night cycle in the *páramo*, in order to check if the presence of anti-freezing activity in the apoplast constitutes a mechanism of tolerance to low temperatures of the tropical high mountain plants.

METHODOLOGY

PLANT MATERIAL

The sampling was done in the Páramo de Palacio located in the Chingaza Natural National Park (Colombia) at two different elevations, 3,300 m.o.s.l. (Valle de los Tunjos) and 3,600 m.o.s.l. (La Mina), between July and September. Mature leaves from three different plants at each site were taken every three hours throughout 24 hour periods. The plants were adult and in good overall conditions. The leaf surface temperature was measured with a K EDL E-Z thermocouple probe. Sampling was done twice in each site.

EXTRACTION

Bits of leaf (1-2 cm^2) were kept in plastic bags at -70 °C for 4 days. They were then moved at room temperature and 50 mM Tris-HCl pH 7.4, 5 mM EDTA and 20 mM ascorbic acid buffer were added. After thirty minutes, the bag contents were gently squeezed to release only the contents of the apoplast. The extracts were centrifuged at 4,000 rpm for 15 minutes at 4 °C and concentrated by lyophilisation. Total protein content was quantified by Bradford's method (1976) as modified by Stoscheck (1990).

UNIDIMENSIONAL SDS-PAGE ELECTROPHORESIS

Lyophilized samples were suspended in 200 µL 5 mM Tris-HCl pH 7.4, 0.5 mM EDTA, 2 mM ascorbic acid buffer; they were centrifuged at 13,000 rpm for 10 minutes. Ten µL of each supernatant were suspended in 5 µL Laemmli sample buffer (Laemmli, 1970). T5% concentration and T12.5% separation gels were used; each well was loaded with 20 µg

protein extract. Electrophoreses were run at 150 V, 30 mA and 10 watts in a Protean cell chamber (BioRad). Gels were then silver stained (Blum *et al.,* 1987). One DScan software (BioRad, version 1.3) was used for determining protein molecular weight. α-lactoalbumin (14.2 kDa), trypsin inhibitor (20.1 kDa), trypsinogen (24 kDa), carbon anhydrase (29 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), egg-white (45 kDa) and bovine albumin (66 kDa; SIGMA) were used as molecular weight patterns.

12:00 h	15:00 h	18:00 h	21:00 h	24:00 h	3:00 h	9:00 h
80	83	65	74	70	81	74
70	62	59	51	64	64	61
63	50	41	47	57	59	55
57	35	36	42	54	42	46
50	31	34	40	41	37	43
49	30	26	39	36	33	39
45	28	22	34	32	30	34
37	27	22	31	25	28	31
36	24	21	28	21	22	26
35	23	19	25	19	20	22
29	22	16	24	15	16	21
26	19	15	20	12	15	20
23	17	14	18		14	19
22			14		13	16
21			13		12	14
20			12			13
17						12
16						
15						
13						

Table 1. Molecular weights of apoplast proteins from *Senecio niveoaureus* leaves collected at 3,300 meters above sea-level. Molecular weights obtained on SDS-PAGE gels and determined by One Dscan software (version 1.3).

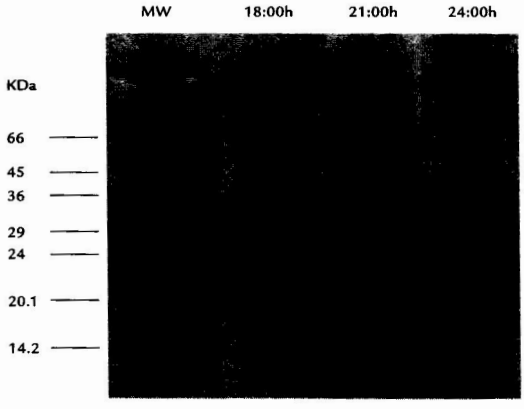

Figure 1. Representative SDS-PAGE of apoplast proteins from leaf protein extracts of *S. niveoaureus* at 3,300 metres above sea-level. Lane 1 protein ladder (MW indicated by numbers), lane 2 sample taken at 18:00h, lane 3 sample taken at 21:00h, lane 4 sample taken at 24:00h. Ten µL of each supernatant were suspended in 5 µL Laemmli sample buffer. T5% concentration and T12.5% separation gels were used; each well was loaded with 20 µg protein extract. Electrophoreses were run at 150 V, 30 mA and 10. Gels were then silver stained. Smear in all the samples was constantly observed due to the presence of thick mucilage.

ANTIFREEZE ACTIVITY ASSAY

The sucrose-sandwich-splat assay based on Worrall *et al.* (1998) and Smallwood (personal communication) was used. 0.5 µg/µL of apoplast extracts of *S. niveoaureus* in 30% (w/w) sucrose was squashed between circular coverslips. The sandwich was dropped into a bath of heptane kept at -80 °C and transferred into a chamber containing heptane at -6 °C. Ice crystals were viewed using a 20X object on an Optiphot microscope (Nikon); images were captured after 30-60 min incubation at -6 °C using a video camera. Recombinant fish AFPIII was used as positive control.

12:00 h	15:00 h	18:00 h	21:00 h	24:00 h	3:00 h	9:00 h
85	80	79	86	127	77	115
76	74	73	83	88	65	93
70	66	64	78	76	54	81
65	60	58	73	62	48	78
58	55	53	68	57	40	73
44	47	46	65	53	37	65
43	42	42	61	48	33	60
42	37	36	56	45	30	54
41	35	33	52	42	29	51
39	32	31	47	38	27	46
36	30	29	42	33	25	42
34	28	26	38	32	23	40
27	26	23	34	29	22	36
25	24	23	33	24	21	35
23	23	21	31	22	19	33
19	22	19	29	19	16	32
18	19	18	26	17	15	31
15	18	16	25		13	27
	16	14	23		12	26
	15	12	22			24
	13		21			24
			18			22
			16			20
						18
						17
						15

Table 2. Molecular weights of apoplast proteins from *Senecio niveoaureus* leaves collected at 3,600 meters above sea-level. Molecular weights obtained on SDS-PAGE gels and determined by One Dscan software (version 1.3).

RESULTS AND DISCUSSION

Electrophoretic patterns of leaf apoplast proteins from *S. niveoaureus* showed the presence of 85 kDa to 12 kDa proteins (Fig. 1). Most of the proteins were in the 35-12 kDa range (Tables 1 y 2; Figs. 2A y 2B). The proteins observed in the 35-12 kDa

molecular range were found mainly when temperatures dropped during the night between 18:00 and 3:00 hrs. and when the temperatures increased during the day between 9:00 and 15:00 hrs. (Fig. 3). More proteins were observed in the material collected at 3,600 m than at 3,300 m (Tables 1 y 2; Figs. 2A y 2B). AFPs described in apoplast from different plant species present molecular weights between 35-11 kDa (Hon *et al.,* 1994; Hon *et al.,* 1995; Griffith and Ewart, 1995; Antikainen *et al.,* 1996; Antikainen and Griffith, 1997; Smallwood *et al.,* 1999), this being in a similar range to that found in *S. niveoaureus* extracts.

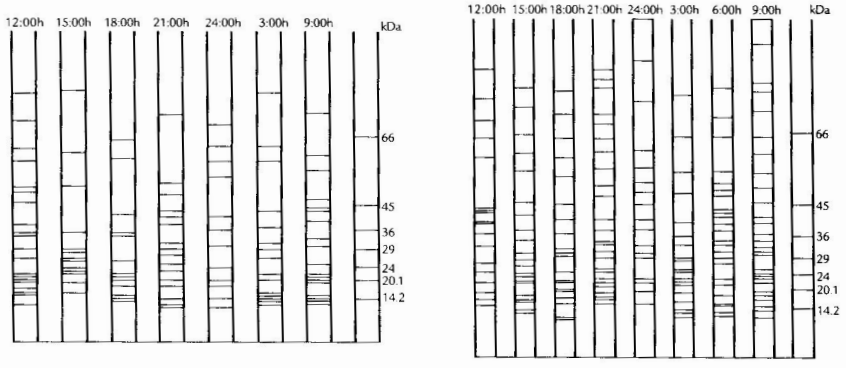

Figure 2A. Molecular weight scheme for *Senecio niveoaureus* leaf protein extracts obtained at 3,300 metres above sea-level. Molecular weights obtained on SDS-PAGE gels and determined by One Dscan software (version 1.3). B. Molecular weight scheme for *Senecio niveoaureus* leaf protein extracts obtained at 3,600 metres above sea-level. Molecular weights obtained on SDS-PAGE gels and determined by One Dscan software (version 1.3).

The analysis of the molecular weights of the apoplast proteins of *S. niveoaureus* leaves, during the day-night cycle and at different elevations showed significant variations among the samples. However, it is not clear if these variations in the electrophoretic patterns are due to the daily variation in temperature during the day-night cycle or to the altitudinal gradient. In fact, some reports have indicated that one limitation of these studies is that they do not demonstrate a cause-and-effect relationship between AFPs and freezing tolerance or winter survival (Griffith *et al.,* 2005).

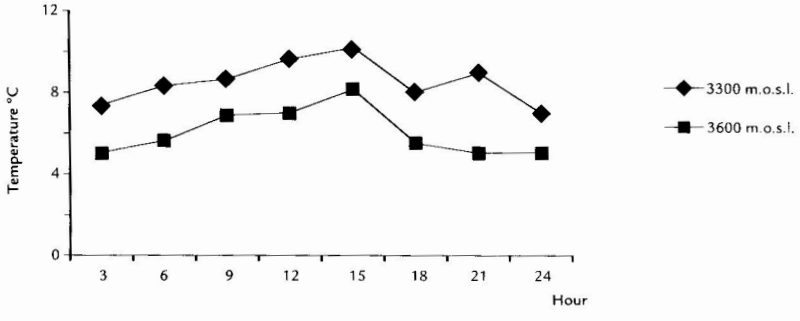

Figure 3. Temperature variation of *S. niveoaureus* leaves at 3,300 and 3,600 metres above sea-level.

To determine the cryoprotective activity of the apoplast extracts, the sucrose-sandwich-splat technique was used. This technique is used for creating small ice-crystals by flash-freezing the solution whose growth and appearance are monitored by microscope (Smallwood *et al.*, 1999).

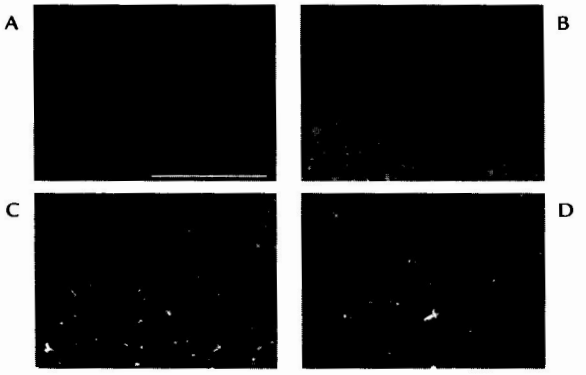

Figure 4. Sucrose-sandwich-splat assay for inhibition of ice re-crystallisation. Object 20X. A. negative control; B. *S. niveoaureus* extract at 3,300 metres above sea-level; C. *S. niveoaureus* extract at 3,600 meters above sea-level; D. recombinant fish AFPIII positive control. Bar represents 125μm.

Figure 4 presents anti-freezing activity results. The appearance of ice-crystals can be observed in a negative control solution which has no anti-freezing activity (Fig. 4A), a positive control solution containing recombinant fish AFPIII having anti-freezing activity (Fig. 4D) and anti-freezing activity induced by low temperature in *S. niveoaureus* leaf extracts (Figs. 4B y 4C). It can be observed that the crystals are smaller in *S. niveoaureus* extracts than in the solution without anti-freezing activity. Moreover, the size of the crystals of *S. niveoaureus* extracts is comparable to that observed in the positive control. The results indicate the presence of AFP-like proteins in *S. niveoaureus* at low temperatures, which could be a mechanism of adaptation of these plants to the extreme environmental conditions of the high Andes. The presence of AFP proteins and activity regardless the temperature or the altitude indicates that *S. niveoaureus* constitutively expresses this type of proteins in order to cope with the wide and unpredictable daily thermal alternation presented in the *páramo*.

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