

## Freezing avoidance in tropical Andean bamboos

Francisca Ely<sup>1,\*</sup>, Sayuri Kiyota<sup>1,2</sup> and Fermin Rada<sup>2</sup>

<sup>1</sup>Instituto Jardín Botánico de Mérida, Facultad de Ciencias, Universidad de los Andes.  
PO Box 343, Mérida 5101, Venezuela.

<sup>2</sup>Instituto de Ciencias Ambientales y Ecológicas, Facultad de Ciencias, Universidad de Los Andes.  
Mérida 5101, Venezuela.

\*Corresponding author's e-mail address: fely@ula.ve

### ABSTRACT

Frost resistance was compared in five species of different life forms in the neotropical woody bamboo genus *Chusquea* growing along a cloud forest-paramo gradient, between 2250–4010 m a.s.l. in the Venezuelan Andes. *C. multiramea* and *C. serrulata* are viny bamboos of the upper montane cloud forest-treeline ecotone (2400–2900 m); whereas *C. angustifolia*, *C. spencei* and *C. guirigayensis* are shrublike bamboos associated to swampy low elevation paramos (2525 m) in the first case, a broad range of paramo ecosystems (2670–3600 m) in the second, and dry high elevation paramos in the third (3800–4010 m). Inter-cellular ice nucleation and 50% tissue injury temperatures were estimated in these five species under laboratory conditions in order to determine frost resistance mechanisms. No significant differences were observed among ice nucleation and tissue injury temperatures in any of these species, indicating that all avoid inter-cellular ice nucleation through supercooling (-12.3 to -10.1 °C). We conclude that variations in supercooling capacity and freezing injury are not related to life form, habitat or elevation and that freezing temperatures are not a determining factor in the altitudinal distribution of tropical Andean bamboos.

**Key words:** supercooling capacity, tropical Andes, *Chusquea*, Bambusoideae, altitudinal gradients.

### INTRODUCTION

Temperature is one of the main environmental factors that affect plant distribution. In the tropical Andes, life-form distribution usually responds to changing environmental conditions along altitudinal gradients, and those that reach the highest altitudes have adapted successfully to resist low temperature environments (Larcher 1995, Körner 1998). In the Venezuelan Andes, low temperature resistance mechanisms have been described for giant rosettes (Goldstein *et al.* 1985), trees (Rada *et al.* 1985, Cavieres *et al.* 2000, García-Núñez *et al.* 2004; Azócar *et al.* 2007, Rada *et al.* 2009), dwarf shrubs (Squeo *et al.* 1991, Azócar 2006), herbs (Azócar *et al.* 1988; Squeo *et al.* 1991, Azócar 2006, Azócar and Rada 2006) and a wide variety of grasses (Márquez *et al.* 2006). Above treeline limits, freezing temperatures may occur any night of the year and surviving below zero

temperatures is achieved either by frost tolerance (withstanding ice formation in inter-cellular spaces) or avoidance (withstanding temperatures well below zero without ice formation in any of the tissues) mainly through deep supercooling (Levitt 1972; Beck *et al.* 1982, 1984, Sakai and Larcher 1987, Pearce 2001). The former has been described as a mechanism utilized in harsher environments with prolonged below zero temperatures, whilst the latter, in less severe environments (Larcher 1995, Körner 1998, Sakai and Larcher 1987). In general, freezing avoidance represents the most common mechanism exhibited by woody species of the tropical high Andes (Squeo *et al.* 1991, Azócar 2006, Azócar and Rada 2006, Rada *et al.* 1985, 2009), given that periods below 0 °C may occur any night of the year, but remain close to zero lasting only a few hours (Azócar and Rada 2006). Avoidance through supercooling in leaves is achieved in tropical Andean giant

rosettes and dwarf shrubs by unique anatomical traits such as compact mesophylls with low apoplastic water contents (Rada *et al.* 1987, Azócar 2006, Azócar and Rada 2006). In other paramo woody species like *Polylepis sericea*, avoidance is achieved by the accumulation of osmotically active solutes and carbohydrates (Rada *et al.* 1985). In contrast with woody life forms, herbaceous plants including grasses, tolerate intercellular ice formation (Squeo *et al.* 1991, Márquez *et al.* 2006). Bamboos regarded as “woody grasses”, are associated to a diversity of high altitude tropical ecosystems, from cloud forests to high elevation paramos (Clark 1995, Monasterio and Molinillo 2003, Clark and Ely 2011). However, how this group responds to freezing temperatures remains unknown. Temperate Japanese bamboos of the genera *Sasa* and *Sasamorpha* avoid frost formation in foliage tissues through deep supercooling, reaching freezing temperatures between -22 and -15 °C (Ishikawa 1984, Sakai 1976, Sakai 1995, Tanaka 2002). In the tropical Andes, all of the high elevation bamboos belong to the genus *Chusquea* (Clark 1989, Clark 1995, Bussman 2004, Niño *et al.* 2006, Clark and Ely 2011), and the greatest species diversity may be found in Andean cloud forests, between 2400-2800 m a.s.l (Clark 1995). At higher altitudes (3000-4000 m a.s.l.), species diversity decreases markedly, and the viny life-form is replaced by a shrublike one. The *Chusquea* species that grow along the upper cloud forest-paramo ecotone are: *C. multiramea* (2200-2750 m a.s.l.), *C. serrulata* (2450-2900 m a.s.l.), *C. angustifolia* (2525-2800 m a.s.l.), *C. spencei* (2780-3650 m a.s.l.) and *C. guirigayensis* (3800-4010 m a.s.l.). The first two species are upper cloud forest viny bamboos whereas; the remaining three species are shrublike and differ in their altitudinal distribution: *C. angustifolia* grows in swampy low paramos, *C. spencei* along the treeline-paramo ecotone, and *C. guirigayensis* exclusively in high elevation paramos.

At present no studies on the effect of freezing temperatures on high altitude Andean bamboos have been conducted. Two main questions are addressed in this study: Firstly, how do Andean bamboos respond to freezing temperatures? One could expect the species of lower altitudes

to be frost avoiders and those of higher elevations frost tolerant. Secondly, does freezing resistance increase in woody bamboos along the altitudinal gradient?

## MATERIALS AND METHODS

Field sample collection and thermal analysis were carried out from 2008 through 2011. Fresh leaf samples were collected at different intervals, during both dry and rainy seasons, along an altitude gradient ranging from 2450 to 4010 m a.s.l., comprising upper cloud forests-treeline ecotones, low and high paramos. Samples of *C. multiramea* and *C. serrulata* were collected at 2450 m a.s.l. in Monte Zerpa cloud forest (08°38' 92" N and 71°24' 63" W). *C. angustifolia* was collected in Las Piñuelas at 2525 m (N 8°37'35" and W 71°24'), which constitutes the lowest paramo of the Venezuelan Andes. *C. spencei*, due to its broad altitudinal range of 930 m (Ely 2009), was collected at three different altitudes: paramos Las Coloradas (N 8°28' and W 71°57') at 2670 m, La Culata (N 08°44' 99" and W 71°04.17') at 3025 m a.s.l., and La Aguada (N 8°35'; and W 71°09') at 3320 m a.s.l. *C. guirigayensis*, was collected in the páramo Piedras Blancas (N 8°53' and W 70°57'), at 4010 m a.s.l. Mean annual rainfall at these five sites are: 2286 mm in Las Piñuelas, 2520 mm in Monte Zerpa, 1877 mm in Las Coloradas, 1780 mm in La Culata, 1573 mm, in La Aguada (Ely 2009) and 800-900 mm in Piedras Blancas (Kiyota 2011).

## Species description

Extensive descriptions of the vegetative characters for these species may be reviewed in Clark (1989), Niño *et al.* (2004), Ely (2009), Kiyota (2011), Clark and Ely (2013). Vouchers of the five species collected are deposited in MERC, Instituto Jardín Botánico de Mérida, Faculty of Sciences, University of The Andes, Mérida, Venezuela.

*Chusquea angustifolia* (Soderstr. and C. Calderón) L.G. Clark. (*F. Ely et al.* 2009, v.s. 44 MERC). Shrub of variable height, 0.2-1.7 m high, that grows between 2520-2800 m a.s.l. Foliage leaves pubescent, somewhat sticky, lanceolate, coriaceous, blades 6-8 cm long x 0.2-5 mm wide.

*Chusquea guirigayensis* Niño, L.G. Clark and Dorr. (*F. Ely et al. 2009*, v.s. 43 *MERC*). Miniature shrub, typically 20-50 cm high (exceptionally 120 cm). In Mérida, this species grows between 3800-4010 m a.s.l. Foliage leaves glabrous, triangular to lanceolate, consistency markedly coriaceous, blades 1-2.5 cm long x 0.3-0.5 cm wide.

*Chusquea multiramea* L.G. Clark and F. Ely. spec. nov. (*F. Ely & Borregales 2006*, v.s. 15 *MERC*). Woody climber, usually 2-6 m high that grows between 2200-2700 m a.s.l. Foliage leaves glabrous, membranous, blades 6.5-8.2 cm long x 3.8-4.2 cm wide.

*Chusquea serrulata* Pilger. (*F. Ely et al. 2006* v.s. 18 *MERC*). Woody climber, usually 3-6 m high, that grows between 2450-2900 m a.s.l. Foliage leaves glabrous, linear, papery blades 18-30 cm long x 0.4-0.6 cm wide.

*Chusquea spencei* Ernst. (*F. Ely et al. 2006* v.s. 1 *MERC*). Shrub, ranging from 0.8-3 m high, growing between 2670-3650 m a.s.l. Foliage leaves glabrous, linear-lanceolate, coriaceous, blades 5-14 cm long x 0.2-0.6 cm wide.

### Climate measurements

Air temperature (°C) and relative humidity (RH) data from the last 10 years were considered, as well as measurements performed at these six localities; from February to October 2008 in Monte Zerpa, La Culata and La Aguada, and from May to November 2010 in Las Piñuelas, El Molino and Piedras Blancas. Data were registered every 15 minutes with portable data loggers at each site (*HOBO, Pro Series*. Onset, Massachusetts, USA). In all of the study sites, sensors were placed at 1.5 m above ground level, protected from direct full sunlight. These were also placed at ground level in the paramos Las Piñuelas, El Molino and Piedras Blancas.

### Thermal analysis

The relationship between tissue ice nucleation and injury temperatures determine frost resistance mechanisms. If these two temperatures coincide well below zero, we refer to freezing avoidance. In frost tolerant plants, ice nucleation temperatures occur close to 0°C while injury at significantly lower temperatures.

Intercellular ice nucleation temperatures were measured in fresh leaves collected at the six sites. A minimum of four trials were carried out in n=18-20 samples per species. Leaf samples were collected from these species during both the dry (January through March) and the rainy (May through September) seasons. Sampling consisted of five young culms per species of different genets collected from culms separated at a minimum distance of 10 m. Culms were cut at ground level in the field, placed in water and the ends cut again under water to avoid formation of air bubbles in xylem vessels (Lei and Koike 1998). In the lab, culms were covered with black plastic bags and allowed to rehydrate overnight. A total of five leaf samples were placed in small glass test tubes, tightly sealed with rubber stoppers in order to avoid tissue moisture changes. Copper-constantan thermocouples were inserted in the tissue samples and temperature was continuously monitored with a 5-channel data logger connected to a PC. Test tubes were immersed in a refrigerated alcohol bath (*NESLAB*, mod. RTE-111). Temperature was then lowered progressively from 5 °C to -25 °C at a rate of approximately 7.5 °C/h and monitored continuously with specially designed software (*Planta-ICAE*). Intercellular ice nucleation was registered through the formation of exotherms, the result of an abrupt increase in temperature generated by heat released during the freezing process.

### Determination of injury temperatures

50% Injury temperatures were determined in these five species through the electrolyte leakage method used previously by Ishikawa (1984) and later modified by Lindén (2002). Electrical conductivity ( $\mu\text{S}$ ) was measured in leaf tissues previously submitted to decreasing low temperatures, from 5 °C to -25 °C, and submerged in deionized water (with an initial electrical conductivity of 0  $\mu\text{S}$ ). Increases in electrical conductivity resulted from electrolyte leakage due to release of potassium ions as a consequence of cell wall rupture. Tissue samples were submitted to the same frost-inducing procedure described previously. This procedure was performed in n=18-20 samples per species. At 5 °C intervals, three tubes were withdrawn from the refrigerated bath, leaf

samples removed from the tubes and placed in clean plastic containers with 15 mm<sup>3</sup> of deionized water. The containers were then refrigerated at 6 °C during 48 h, and electrical conductivity was measured with an ExStik digital conductimeter, mod. EC500 (*Extech Instruments, U.S.A.*). After measurements were performed, complete tissues rupture was induced by submerging samples briefly in liquid nitrogen and placing them again in their respective containers. These were refrigerated again for 48 h and electrical conductivity was measured afterwards. This last measurement corresponded to the electrical conductivity of samples after 100% leakage had occurred. Tissue injury was estimated as the temperature at which 50% leakage occurred through the following equation:

$$T_{50\%} = \frac{\text{Initial Electrical Conductivity} * 100}{\text{Final Electrical Conductivity}}$$

Initial Electrical Conductivity corresponded to the temperature to which the tissue was exposed before withdrawing the tubes from the refrigerating bath, whereas Final Electrical Conductivity represented the electrical conductivity of the samples after inducing complete tissue rupture.

### Data analysis

Ice nucleation temperatures were represented graphically in box plots (*Sigma Plot, ver. 10.0*), and average temperatures and standard deviation errors were estimated. U Mann-Whitney

non-parametric tests were carried out to determine whether or not differences between intercellular ice nucleation and injury temperatures were statistically significant for each species. Kruskal-Wallis tests (*SPSS Statistics, ver. 17.0*) were performed to determine statistically significant differences regarding ice nucleation and injury temperatures among these five species.

## RESULTS

### Climate characteristics

Below zero temperatures occurred only above 3000 m, and were relatively infrequent at treeline limits (3025-3320 m) where they remained close to zero, being more frequent in the upper open paramo limits (3800-4010). The lowest night temperatures were registered in Páramo de Piedras Blancas followed by Páramo La Aguada (Table 1). The frequency of nights with freezing temperatures increased during the dry season (end of December through the end of March 2008) above 3000 m, representing a 10% of the total of days registered at 3000 m, 18% at 3320 m, and 41% at 4010 m.

### Intercellular ice nucleation and injury temperature

Both ice nucleation and 50% injury temperatures were consistent in all of the trials. In these five species, average intercellular freezing temperatures varied between -12.1 and -10.1 °C, whereas average 50% injury values

Table1. Average air temperatures registered at the six study sites. Maximum and minimum temperatures registered are indicated in parentheses.

Study site	Average Temperature (°C)		Average Min. temperature (°C)		Average Max. temperature (°C)	
	Soil	Air	Soil	Air	Soil	Air
Monte Zerpa (2450 m)	ID	13.02	ID	8.03 ± 0.01 (6.53)	ID	17.06 (20)
Las Piñuelas (2525 m)	13 ± 0.03	14 ± 0.03	10 ± 0.2 (6.6)	8.5 ± 0.1 (4.6)	25 ± 0.6 (32)	20 ± 0.2 (25)
Las Coloradas (2670 m)	11 ± 0.02	12 ± 0.02	7.5 ± 0.2 (4.6)	9.0 ± 0.1 (7.0)	15 ± 0.2 (19)	17 ± 0.2 (21)
La Culata (3025 m)	ID	9.25 ± 0.07	ID	4.3 ± 0.65 (-0.16)	ID	18.8 ± 0.08 (19)
La Aguada (3320 m)	ID	7.6 ± 0.1	ID	2.3 ± 1.0 (-0.68)	ID	20 ± 0.06 (24)
Piedras Blancas (4010 m)	8.6 ± 0.1	6.2 ± 0.04	1.5 ± 0.1 (-12)	2.0 ± 0.1 (-4)	27 ± 1.0 (46)	15 ± 0.4 (21)

ID: insufficient data due to technical difficulties with the loggers

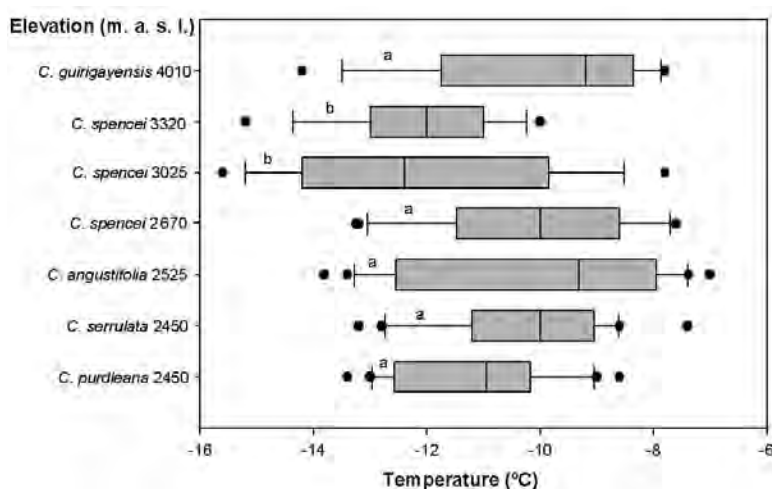


Figure 1. Relationship between ice nucleation temperatures and altitude for five *Chusquea* species along the 1560 m altitudinal gradient. Black circles represent extreme (high and low) values of ice nucleation temperatures in each case. Different letters depict significant differences ( $p < 0.05$ ).

ranged between  $-12.3$  and  $-10.3$  °C (Table 2). No statistically significant differences were observed between intercellular ice nucleation temperatures (exotherm formation temperature) and injury temperatures in any of the species along the altitudinal gradient, indicating that in these five *Chusquea* species, intercellular ice nucleation was avoided through a moderate supercooling capacity.

Intercellular ice nucleation temperatures did not decrease linearly along the altitude gradient in this genus (Figure 1); nor did they vary significantly between the two viny cloud forest species (*C. multiramea* and *C. serrulata*); nor amongst the latter and the paramo shrublike species growing at the lower and upper limits of this gradient (*C. angustifolia* at 2520 m, the genets of *C. spencei* at 2670 m, and *C. guirigayensis* at 3800–4010 m). However, significant differences were observed between the genets of *C. spencei* growing at lower and upper limits of its distribution range (2670 m vs 3025–3320 m), as well as between the genets of *C. spencei* growing above 3000 m and the remaining four species (Figure 1, Table 2).

## DISCUSSION

*Chusquea* is distinguished as the genus with the greatest diversity regarding species, habitats, life forms and altitudinal distribution in tropical Andean ecosystems (Clark 2001), yet above 3000 m, species diversity decreases markedly.

These variations in species abundance suggest that freezing temperatures could be a determining factor in woody bamboo distribution in tropical mountain ecosystems. We had initially assumed that the bamboos growing below

Table 2. Average intercellular ice nucleation and 50% injury temperatures measured in these five *Chusquea* species. Maximum and minimum temperatures are indicated in parenthesis.

Species/ elevation (m a.s.l.)	Intercellular ice nucleation temperature (°C)	50% Injury temperature (°C)
<i>C. multiramea</i> (2450 m)	(-9.0) $-11.0 \pm 0.3^a$ (-13.4)	(-10.8) $-11.5 \pm 0.3^a$ (-12.4)
<i>C. serrulata</i> (24500 m)	(-9.0) $-10.5 \pm 0.3^a$ (-13.0)	(-9.6) $-10.1 \pm 0.4^a$ (-11.9)
<i>C. angustifolia</i> (25250 m)	(-7.4) $-10.8 \pm 0.2^a$ (-13.8)	(-11.4) $-12.3 \pm 0.3^a$ (-13.1)
<i>C. spencei</i> (26700 m)	(-7.6) $-10.6 \pm 0.4^a$ (-13.3)	(-8.4) $-10.2 \pm 0.4^a$ (-13.7)
<i>C. spencei</i> (30250 m)	(-7.9) $-12.1 \pm 0.5^b$ (-15.6)	(-9.8) $-12.0 \pm 0.2^b$ (-13.5)
<i>C. spencei</i> (33200 m)	(-10.4) $-12.0 \pm 0.3^b$ (-15.0)	(-9.8) $-12.2 \pm 0.3^b$ (-15.9)
<i>C. guirigayensis</i> (4010 m)	(-8.0) $-10.3 \pm 0.4^a$ (-14.2)	(-9.0) $-10.6 \pm 0.3^a$ (-12.0)

Different letters depict significant differences ( $p < 0.05$ ).

treeline limits (3000 m), which are not exposed to freezing temperatures, such as the viny species (*C. multiramea* and *C. serrulata*) and the genets of *C. angustifolia* and *C. spencei* of low paramo ecosystems (2520 and 2670, respectively) were altogether devoid of freezing resistance mechanisms in which case, ice nucleation and injury temperatures should have occurred very close to 0 °C, as described for other woody species of the upper cloud forest-paramo ecotone (Cavieres *et al.* 2000). Nevertheless, all five species avoided intercellular ice formation through a moderate supercooling, regardless of their life-form, habitat, plant height, foliage leaf size and consistency. Supercooling capacity values were higher in these *Chusquea* species than those reported for other paramo woody species (Rada *et al.* 1985, 2009; Cavieres *et al.* 2000), and comparable to those reported for giant rosettes of the genus *Espeletia* (Goldstein *et al.* 1985, Rada *et al.* 1987). Another unexpected result was that neither intercellular ice nucleation, nor 50% injury temperatures varied significantly between the species situated at the upper and lower limits of the cloud forest-paramo gradient (2450 and 4010 m). Only *C. spencei* presented a slight increase in its supercooling capacity with increasing altitude, as the differences between the genets growing below and above 3000 m suggest. A possible explanation for the relatively uniform supercooling capacity observed in these five species is that after the last glaciation, cloud forest and paramo boundaries suffered repeated displacements, with paramo ecosystems descending as low as 2000 m (Van der Hammen 1974, 1988, Van der Hammen 2000, Salgado-Labouriau *et al.* 1977, 1992). In addition, minimum air temperatures have also increased considerably during the last decades, reducing the frequency of nocturnal frosts, as recent microclimate studies indicate (Monasterio and Reyes 1980, Azócar and Rada 2006, Azócar 2006, Ely 2009, Kiyota 2011).

Our results suggest that neither plant height nor elevation necessarily condition freezing resistance mechanisms in this group. *C. guirigayensis* with the smallest height and growing at the highest elevations responds to freezing temperatures in the same manner as the other species studied. Freezing resistance mechanisms differ among herbaceous tussock grasses and strongly lignified grasses such as bamboos,

regardless of whether they grow in temperate (Ishikawa 1984, Tanaka, 2002, Ashworth and Pearce 2001, Liu and Osborne 2008) or tropical climates (Márquez *et al.* 2006). Tussock grasses of the Venezuelan paramos tolerate extracellular freezing, with intercellular ice nucleation temperatures between -6.3 and -3 °C, and 50% injury of foliage tissues occurring between -18 and -9.8 °C (Márquez *et al.* 2006). Tussock grasses are subjected to lower temperatures and for longer intervals; due to their proximity to the ground where the temperatures are lowest in the air-soil gradient, therefore foliage tissues are exposed to freezing temperatures in their early development, in contrast with woody bamboos, in which developing organs are protected from extreme temperatures by thick culm leaves until they have reached maturity (Ely 2009). In the Venezuelan paramos, where freezing air temperatures typically remain close to 0 °C and last for only a few hours, a moderate supercooling capacity combined with the protection of developing organs should be sufficient to impede the formation of intercellular frost in foliage tissues.

These results support the studies conducted in the Japanese species of *Sasa* and *Sasamorpha*, which differ from these *Chusquea* species in their greater supercooling capacity (-22 to -15 °C, Sakai 1976, 1995, Ishikawa 1984), which likely evolved as an adaptation to the seasonal winters (Tanaka 2002). Based on these results we also conclude that the altitudinal distribution of *Chusquea multiramea*, *C. serrulata*, *C. angustifolia*, *C. spencei* and *C. guirigayensis* in the Venezuelan Andes is not conditioned by freezing temperatures, but more likely by other environmental factors not taken in account in the present study.

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Figure 2. *Chusquea multiramea*



Figure 3. *Chusquea spencei*



Figure 4. *Chusquea guirigayensis*



Figure 5. *Chusquea spencei*



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