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SUPERCOOLING CAPACITY INCREASES FROM SEA LEVEL TO TREE LINE IN THE HAWAIIAN TREE SPECIES *METROSIDEROS POLYMORPHA*

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Population-specific differences in the freezing resistance of *Metrosideros polymorpha* leaves were studied along an elevational gradient from sea level to tree line (located at ca. 2500 m above sea level) on the east flank of the Mauna Loa volcano in Hawaii. In addition, we also studied 8-yr-old saplings grown in a common garden from seeds collected from the same field populations. Leaves of low-elevation field plants exhibited damage at −2°C, before the onset of ice formation, which occurred at −5.7°C. Leaves of high-elevation plants exhibited damage at ca. −8.5°C, concurrent with ice formation in the leaf tissue, which is typical of plants that avoid freezing in their natural environment by supercooling. Nuclear magnetic resonance studies revealed that water molecules of both extra- and intracellular leaf water fractions from high-elevation plants had restricted mobility, which is consistent with their low water content and their high levels of osmotically active solutes. Decreased mobility of water molecules may delay ice nucleation and/or ice growth and may therefore enhance the ability of plant tissues to supercool. Leaf traits that correlated with specific differences in supercooling capacity were in part genetically determined and in part environmentally induced. Evidence indicated that lower apoplastic water content and smaller intercellular spaces were associated with the larger supercooling capacity of the plant's foliage at tree line. The irreversible tissue-damage temperature decreased by ca. 7°C from sea level to tree line in leaves of field populations. However, this decrease appears to be only large enough to allow *M. polymorpha* trees to avoid leaf tissue damage from freezing up to a level of ca. 2500 m elevation, which is also the current tree line location on the east flank of Mauna Loa. The limited freezing resistance of *M. polymorpha* leaves may be partially responsible for the occurrence of tree line at a relatively low elevation in Hawaii compared with continental tree lines, which can be up to 1500 m higher. If the elevation of tree line is influenced by the inability of *M. polymorpha* leaves to supercool to lower subzero temperatures, then it will be the first example that freezing damage resulting from limited supercooling capacity can be a factor in tree line formation.

Keywords: supercooling, freezing resistance, *Metrosideros polymorpha*, tree line, Hawaii, nuclear magnetic resonance, plasticity.

Introduction

Physiological and morphological traits related either to avoidance or tolerance of extracellular tissue freezing have been observed in plants growing at high elevation in tropical regions where nocturnal freezing temperatures are frequent. In the Venezuelan Andes, for example, freezing avoidance by supercooling (tissue cooling below the equilibrium freezing temperature without ice formation) to −15°C is common in leaves of giant rosette plants that are growing above the tree line (Goldstein et al. 1985; Rada et al. 1987). Although leaves of these giant rosette plants supercool, other tissues in the same plants avoid exposure to freezing temperatures through thermal insulation (Goldstein and Meinzer 1983; Squeo et al. 1991).

Freezing tolerance (the ability of tissue to tolerate extracellular ice formation without permanent damage) has been observed in *Draba chianophila* miniature rosettes in the high Andes (Azocar et al. 1988), in *Lobelia* and *Senecio* (giant rosettes from equatorial mountains of Africa; Beck et al. 1984), and in several tropical alpine Hawaiian species (Lipp et al. 1994). *Polylepis sericea*, a neotropical high-elevation tree species, increased nocturnal production of low–molecular weight solutes in response to minimum night temperatures, resulting in a 3°C decrease of the equilibrium freezing temperature

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(Rada et al. 1985). Therefore, many different types of adaptations that allow the plant to cope with subzero temperatures have been described for high-elevation tropical plants.

Metrosideros polymorpha Gaud. (Myrtaceae), the dominant endemic tree species in the Hawaiian Islands, exhibits an extreme polymorphism across its wide ecological range, and particularly along elevation gradients from sea level to its high-elevation limit, which on the Mauna Loa volcano is at tree line, located at ca. 2500 m above sea level (Mueller-Dombois and Fosberg 1998). Morphological variations are associated with substrate age, substrate type, and elevation (Corn and Hiesey 1973; Stemmermann 1983; Dawson and Stemmermann 1990; Vitousek et al. 1992). Trees with pubescent leaves are typically found on young lava flows (those that are <300 yr old) and above 2000 m elevation. Larger trees with glabrous leaves are found at lower elevations and on older lava flows (>2500 yr old), where soils are better developed. Young lava flows and elevations above the persistent tropical inversion layer (which oscillates frequently between 1800 and 2400 m above sea level [Giambelluca and Nullet 1991]) are characterized by both low soil moisture availability and low soil nitrogen content (Leuschner and Schulte 1991; Vitousek et al. 1992). There is only one tree species, *Sophora chrysophylla*, growing in a small open forest stand up to 2900 m above sea level on relatively well-developed soils on Mauna Kea volcano, upslope from the *M. polymorpha* tree line (Leuschner 1996; Mueller-Dombois and Fosberg 1998).

Freezing temperatures tend to be frequent at around 2500 m above sea level, particularly during the months from December to March. *Metrosideros polymorpha* growing at high elevations are exposed to these harsh conditions, any of which can affect the growth and physiological performance of this plant. In this study, the effects of subzero temperatures on leaves of *M. polymorpha* were investigated. This study was initiated because planting efforts to reestablish this species in the high-elevation grasslands of Hakalau Forest National Wildlife Refuge, located on the windward slopes of Mauna Kea (island of Hawaii), failed (i.e., mortality reached almost 100% within 2 yr of planting; Scowcroft and Jeffrey 1999; Scowcroft et al. 2000). Symptoms of freezing damage were observed during winter months, which led us to suspect that nocturnal radiative frost could have been a factor in the high mortality.

The primary objectives of this study were to identify both the type of freezing resistance mechanism of *M. polymorpha* populations growing from near sea level to tree line and the temperatures at which irreversible freezing damage occurred in leaves along this elevational gradient. A secondary objective was to determine whether differences in the latter were genetically or environmentally determined. To meet this objective, we compared the results of freezing experiments with field and common garden plants. This information, along with information on the temperature lapse rate and seasonal changes of air and soil temperatures, was expected to give us a better understanding of the functional significance of elevational trends in leaf morphology and anatomy observed in this highly polymorphic species and of the environmental and plant factors that determine the upper limits of *M. polymorpha* distribution on the Hawaiian volcanoes.

Material and Methods

Site Characteristics and Air Temperature Measurements

Metrosideros polymorpha trees growing at three elevations (107, 1280, and 2470 m) and two different age flows per elevation were studied on the east-facing (windward) slope of Mauna Loa on the island of Hawaii. Mauna Loa is an active shield volcano comprising primarily basaltic lavas. The surface flows have been extensively dated, described, and mapped (Lockwood et al. 1988). Sites that had *M. polymorpha* trees growing on both young (113–148 yr) and old (>2500 yr) flows were chosen at each elevation. In all sites, *M. polymorpha* is the dominant species, particularly at higher elevations, where it composes 100% of the canopy trees. The canopy trees at sea level are ca. 5–7 m tall, and at tree line, these trees are ca. 3–4 m tall. Leaf size decreases with elevation from ca. 11.5 cm^2 at sea level to 4 cm^2 at 2470 m above sea level. Leaves are typically horizontal at all elevations; however, petiole and internode lengths decrease significantly with increasing elevation (Cordell et al. 1998).

The pattern of precipitation is dominated by orographic lifting of prevailing northeasterly winds and is strongly influenced by the trade-wind inversion (Giambelluca and Sanderson 1993). Mean annual rainfall increases from <4000 mm at sea level to ca. 6000 mm at 760 m elevation and then decreases with elevation to ca. 1500 mm at the upper limit of *M. polymorpha* (2500 m above sea level; Giambelluca et al. 1986). Mean annual temperature decreases with elevation from ca. 23°C at sea level to 7°C at 3400 m elevation (Nullet and Sanderson 1993).

Air temperatures were obtained from the National Weather Service, Western Regional Climate Center, from seven high-elevation meteorological stations in Hawaii. Six stations contained 43–49 yr of continuous temperature records, and one station (Haleakala Summit) contained 18 yr of continuous records. In addition, monthly minimum air temperature, measured at 2 m above the soil, and soil surface temperature, measured with an infrared thermometer (Everest Interscience, Fullerton, Calif.), were recorded at a 2470-m elevation site with a 21X datalogger (Campbell Scientific, Logan, Utah) that was located on the windward side of Haleakala volcano (Maui) for a period of ca. 6 yr.

Temperature profiles were determined on Mauna Kea in an open area at Hakalau forest at an elevation of 1980 m. Air and soil surface temperatures were monitored from February through April 1993 with fine-wire (24-gauge) copper-constantan thermocouples connected to a 21X datalogger (Campbell Scientific). Air temperatures were measured every 30 s with shaded thermocouples located at 5, 40, 80, and 200 cm above the soil, and these air temperatures were averaged every 15 min.

Common Garden Plants

In October 1991 and February 1992, *M. polymorpha* seeds were collected from 10 sites along the Mauna Loa gradient and sown in a cinder-topsoil mixture at the Hawaii Volcano Experiment Station located in Volcano, Hawaii (1190 m above sea level). Upon seed germination, pots were weeded to one plant per 6-L pot. A minimum of 25 pots from each population

were randomly placed in each of three blocks in an open one-sided plastic-covered greenhouse, and these pots were subsequently moved out into the open after several years. All plants were watered daily and fertilized quarterly with time-release pellets. Of the 10 seed source populations along the elevational gradient, three that corresponded to the field populations described above were chosen for use in this study: 107, 1280, and 2470 m above sea level. The age differences between the common garden and field plants may have confounded the interpretation of our results, but previous studies have shown that the phenotypic expression of morphological traits in *M. polymorpha* seedlings are fixed within 2 yr (Cordell et al. 1998).

Ice Nucleation Temperature

Ice nucleation temperatures (temperatures immediately before freezing occurs) were obtained in laboratory experiments. Branches ca. 30–50 cm in length were excised from the field and from common garden plants at dawn, enclosed in plastic bags, placed in a cooler, and transported immediately to the laboratory for thermal analysis studies. Large branches were used for thermal analysis because excised leaves may freeze at lower temperatures than will leaves of intact plants (Boorse et al. 1998). Branches were placed between two heat exchangers in a Styrofoam box. A solution of 45% water and 55% ethylene-glycol (v/v) was circulated through the heat exchangers, and the temperature was adjusted using a refrigerated bath (RTE -111, Neslab, Newington, N.H.). A total of 10 branches were collected from different individuals in the field (five from each substrate age) at each elevation, and 10 branches were collected from common garden plants (five parents from each substrate age from three elevations).

Leaf temperature was lowered at ca. 10°C/hr from ambient to −18°C (this rate is similar to those used in other studies; Levitt 1980; Nobel 1981; Goldstein and Nobel 1991). The air and leaf surface temperatures were monitored at 3-s intervals using 24-gauge copper-constantan thermocouples connected to a datalogger (CR10X, Campbell Scientific). The thermocouples were placed in contact with the leaf tissue and held in place using small pieces of surgical tape. Termination of supercooling was indicated by a rapid increase in temperature, which represented the heat of fusion of water following ice nucleation.

Freezing Injury

Leaves collected from field-grown and common garden plants were screened for tissue damage by measuring electrolyte leakage from leaf tissues (Wilner 1960). Leaves of the same whorl (recently fully expanded mature leaves) and size were selected from each individual and placed into small airtight plastic bags. The air was removed from the bags to avoid tissue dehydration. These bags were placed inside a water bath, and the temperature was decreased from ambient to −15°C. At ca. every 2.5°C, the temperature was held constant for 20 min, and then one set of bags was removed from the water bath. Retrieved leaves (still in their bags) were allowed to equilibrate at room temperature. The leaves were then removed from the bags, and a 2-cm^2 bore was taken from the center of each leaf. After the cut edges were lightly blotted with a tissue, the samples were weighed and placed into small scintillation vials (which were filled with 10 mL of deionized water) and shaken at ambient temperature for 14 h. After shaking, the leaf material was removed from the vials, and the electrolyte conductivity of the solution was measured with a conductance meter (Model 32, Yellow Springs Instrument, Yellow Springs, Ohio). Additional leaf samples from each population were also placed in the small airtight plastic bags for determination of minimum electrolyte leakage at ambient temperature and of maximum electrolyte leakage at −70°C. These samples were maintained at their two respective temperatures for 2 h before the rest of the above protocol was implemented.

Leaf Characteristics, Osmotic Potential, and Relative Water Content

Leaf samples were collected from both the field and the common garden plants. Five individuals were randomly selected from each population. Fully expanded sun leaves were selected from the most recent flush; 5 × 5-mm sections were cut from the middle of the leaf and placed in a fixative composed of 10% dimethyl sulfoxide, 1% Tween 20, and 2% paraformaldehyde (pH 7.4). Samples were placed in a vacuum for 2 h and stored under refrigeration. The samples were frozen to −20°C and sectioned with a Leica cryocut 1800 (Reichert-Jung, Nussloch, Germany). Transverse sections were used to determine the thickness of the lamina using a light microscope. Leaf pubescence weight was determined using procedures described by Joel et al. (1994). Ten pairs of opposite leaves were collected from each individual, and leaf surface area was determined using a Delta-T leaf-area meter (Delta-T Systems, Cambridge). One leaf was used as a control, and the leaf pubescence was removed from the opposite leaf. The pubescence was scraped with a scalpel, and care was taken to only remove the pubescence layer without harming the other tissues. It was assumed that the two opposing leaves had the same amount of pubescence. Leaves were then oven-dried at 70°C for determination of leaf mass per unit area (LMA). The weight difference between the control LMA and the shaved LMA represents the amount of pubescence of the leaves.

The osmotic potential of leaf tissue samples obtained from field and common garden plants was measured with a vapor pressure osmometer (Wescor 5500, Logan, Utah). The leaves to be sampled were covered with plastic bags, removed from the plant, placed into a cooler containing ice, and transported to the laboratory to be placed in a deep freezer at −70°C for several days. Subsequently, leaf tissue samples were placed within a rubber tube and crushed by a vise to release sap. A 0.01-mL aliquot of leaf sap was then collected with a pipette, placed on small filter disks, and promptly inserted into the vapor pressure osmometer for osmotic potential determinations. The water content ([fresh weight − dry weight]/dry weight) of field and common garden plants was determined by measuring the fresh weight of leaves collected early in the morning and the dry weight after drying leaves at 70°C for 5 d.

Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) studies were conducted to noninvasively determine the liquid water content (during

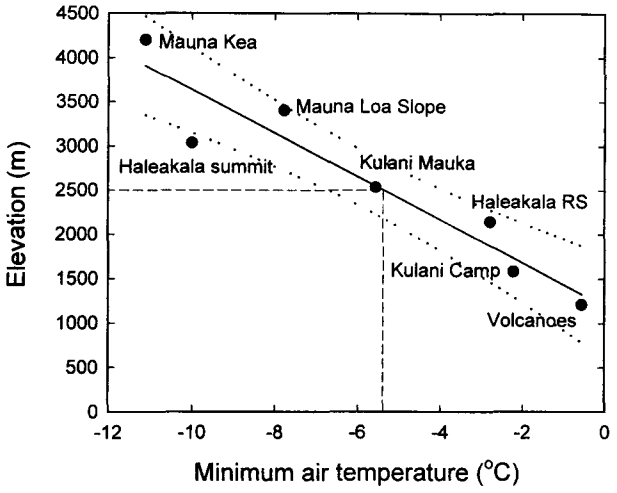

Fig. 1 Absolute minimum air temperatures at 1.5 m above the ground in relation to elevation; data were obtained from high-elevation meteorological stations in Hawaii. The linear regression $y = -244.30x + 1190.4$, $r^2 = 0.90$ (solid line) and the 95% confidence interval (dotted line) are shown. The dashed line indicates the predicted absolute minimum air temperature at 2500 m elevation, corresponding to the *Metrosideros polymorpha* tree line.

freezing) of leaf tissue samples collected from *M. polymorpha*. All spectra were recorded on a GN-Omega-500 NMR spectrometer operating at 500.112 MHz for ^1H. The liquid water content was measured as a function of temperature, and the relaxation rates, described below, were measured at 0°C. A water reference sample, with a volume similar to that of the leaf sample to be analyzed, was used to adjust the homogeneity. The temperature of the leaf sample was measured with a copper-constantan thermocouple positioned in the sample compartment at ca. 3 mm below the sample tube. The temperature control unit was calibrated with a standard methanol sample and was accurate to 0.1°C.

Six individual leaves of similar size and age (i.e., position along the stem) were used, three from a low-elevation and three from a high-elevation population. Approximately 1 g of fresh leaf material, consisting of whole leaves of *M. polymorpha*, was placed in the sample tubes. The sample was first equilibrated at 10°C, and then the temperature was lowered in steps of 3°–5°C, down to −20°C. Each sample was allowed to equilibrate for a minimum of 20 min at each experimental temperature, after the temperature controller indicated that the set point had been reached. The radio-frequency circuit of the sample probe was readjusted before the next spectrum was recorded. All spectra were recorded using quadrate detection and are the sum of eight scans.

The raw free induction decays were baseline corrected (to remove any remaining bias in the receiver) and Fourier transformed, and the phase was adjusted to yield an adsorption mode frequency spectrum. All samples studied yielded asymmetric peak shapes for the water signal, indicating that the observed spectrum was the result of the number of unresolved resonances. Consequently, these peaks were deconvoluted in their individual component signals using the spectral analysis package available in the GN-Omega operating software (version 6.0.2).

The room-temperature spectrum from each sample was used as a starting point for subsequent spectra obtained from the experimental temperature series. The experimental spectrum could be adequately represented using a set of two Lorentzian lines. Each line or peak from the sample can be described by three parameters: the signal position in the spectrum, its amplitude, and the width of the line at one-half the maximum peak height. For overlapping lines it was necessary to simulate the observer signal by using a computer-generated spectrum that is a sum of individual peaks. From this simulated spectrum, systematic variation via a computer fitting program was used to best approximate the observed spectrum. Iterative analysis was most easily accomplished by varying pairs of parameters for each line in succession. Liquid water content of the sample was determined from the area under each line or peak in the spectrum and is represented as a percentage of the maximum liquid water content at 10°C. The sum of both spectrums was used in the final analysis.

In addition to liquid water content determinations at different temperatures, relaxation rates were obtained with NMR studies in order to assess the properties of water in the samples (i.e., degree of water binding and mobility of water protons). Spin-lattice relaxation rates (1/relaxation time, T_1^{-1}) were determined at 0°C using the standard inversion-recovery sequence (Carr and Purcell 1954). The spin-spin relaxation rates (T_2^{-1}) were determined using the Carr-Purcel-Meiboom-Gill method (Meiboom and Gill 1958), which uses a delay between successive π pulses in the pulse train of 110 μs. Relaxation experiments incorporated composite pulses that compensated for resonance offset effects (Freeman et al. 1980). All relaxation rates were determined by fitting a single exponential function to the experimental data using the least-squares routines available in the software of the spectrometer. The relax-

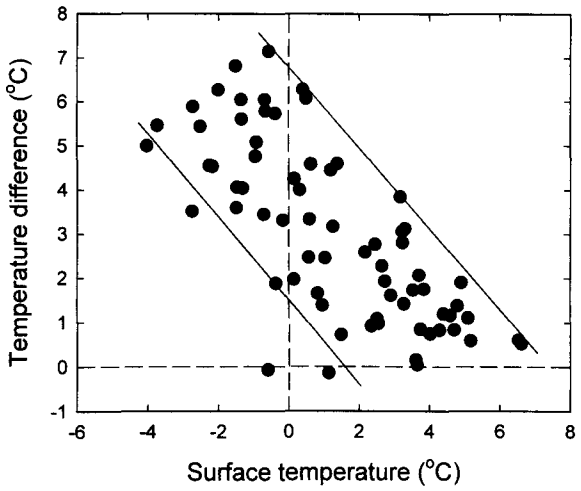

Fig. 2 Difference between minimum monthly air temperatures (measured at 2 m above the ground) and the surface temperature ($T_{air} - T_{surface}$) as a function of surface temperature for a 2470-m elevation site located on the windward side of Haleakala volcano, Maui, Hawaii. The data set corresponds to ca. 6 yr of measurements. The boundary lines of the data set are shown.

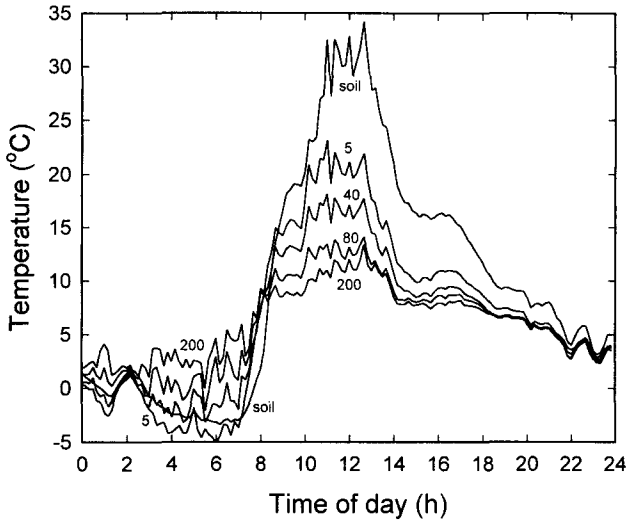

Fig. 3 Diurnal courses of soil temperature (*soil*), measured 0.5 cm below the surface, and air temperatures measured 5, 40, 80, and 200 cm above the soil surface. Measurements were made at 1980 m above sea level at the Hakalau Forest National Wildlife Refuge (island of Hawaii) on February 2, 1994.

ation rates were calculated for each one of the deconvoluted spectra, each one representing a different leaf tissue water fraction (i.e., apoplastic and symplastic water fractions).

Results

Air Temperatures

Extreme minimum air temperatures at 1.5 m above the ground from high-elevation meteorological stations on Mauna Loa, Mauna Kea, and Haleakala, the three largest Hawaiian volcanoes, are shown in figure 1. The data indicate that minimum air temperatures are lower than −5.3°C above 2500 m above sea level (the upper limit of *Metrosideros polymorpha*) and the lapse rate is ca. 4.4°C per 1000 m of elevation. The minimum monthly air temperatures and the corresponding surface temperatures measured at 2470 m elevation on the windward slope of Haleakala during a 6-yr period indicated that nighttime surface temperatures can be 4°C lower than air temperatures (fig. 2). Although subzero air temperatures are rare at the site, surface temperatures of below −4°C are not uncommon. Temperature data collected during the winter of 1994 at 1980 m elevation also indicated that sensors at 2.0 m above the ground failed to detect subzero temperatures. Air temperature near the soil surface, however, dropped to −5°C at night (fig. 3).

Ice Nucleation Temperatures and Freezing Injury

Representative freezing exotherms for low-, intermediate-, and high-elevation *M. polymorpha* leaves collected from plants growing in the field and in the common garden are depicted in figure 4. Ice nucleation occurred below the equilibrium freezing temperature (assumed to be close to −1.5°C according to the Van't Hoff's equation and osmotic potential determinations of leaf tissues) in all cases. Leaves from high-elevation field and common garden plants had the lowest ice nucleation temperatures (table 1). The area under the exotherm reflects the amount of water available for freezing (fig. 4). Accordingly, leaves from the high-elevation population have relatively low amounts of water compared with leaves from lower elevations.

Electrical conductivity, a measure of irreversible cell damage, increased rapidly below 0°C for the low-elevation field plants and reached the maximum at ca. −6°C, which indicates that the leaves of low-elevation *M. polymorpha* are very sensitive to subzero temperatures (fig. 5). The electrical conductivity of the high-elevation field plants, on the other hand, remained low until ca. −8°C and then increased rapidly, which indicates that damage does not occur at mild subzero temperatures. Leaves from twigs collected from plants growing on young lava flows (which tended to be pubescent) exhibited damage at lower temperatures than did leaves from plants growing on old lava flows (predominantly glabrous). Tissue damage thresholds for common garden plants, depicted by a rapid increase in electrical conductivity, occurred at similar temperatures (ca. −5°C), and tissue damage thresholds were independent of seed source population (fig. 5).

The ice nucleation temperature of leaf tissues from field populations decreased from −5.8° to −8.7°C with increasing elevation (table 1). The ice nucleation temperature of common

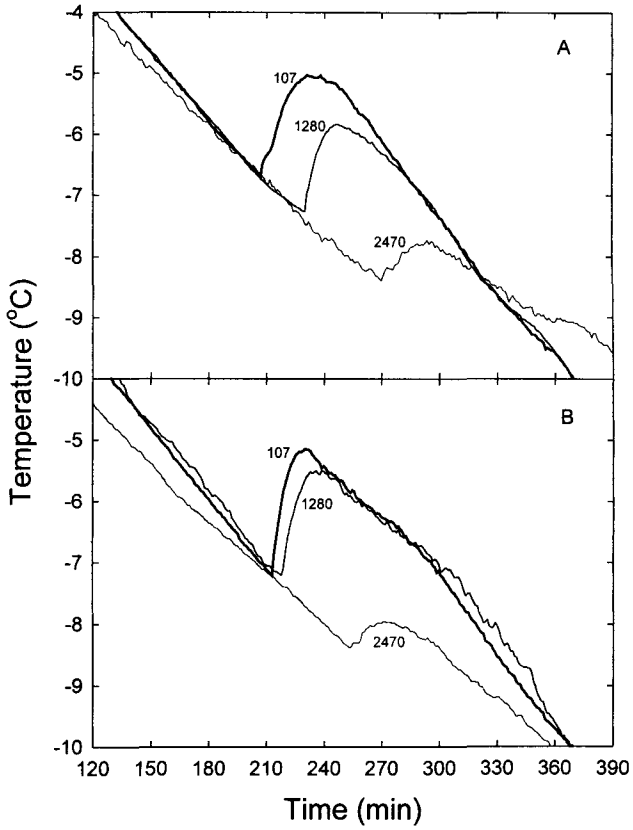

Fig. 4 Representative exotherms for *Metrosideros polymorpha* leaves collected from plants growing at three different elevations (107, 1280, and 2470 m) in the field (*A*) and from common garden plants whose seed source was from the same field populations (*B*).

Table 1

Summary of Anatomical, Morphological, and Physiological Characteristics of *Metrosideros polymorpha* Leaves Collected from Plants Growing at Three Different Elevations in the Field and from Common Garden Plants Whose Seed Source Was from the Same Populations as the Field Plants

Location and elevation (m)	Substrate age	Lamina thickness (μm)	LMA (g/m^2)	Pubescence (g/m^2)	LWC (%)	Osmolarity (mmol/kg)	Ice nucleation (°C)	LT_{50} (°C)
Field:								
107	Young	304.8 (22.5)	110 (5.5)	14.7 (7.9)	155 (12)	490 (30)	−6.2 (0.4)	−2.5
	Old	320.6 (15.4)	120 (8.0)	12.2 (3.8)	140 (12)	510 (20)	−5.8 (1.0)	−1.3
1280	Young	434.4 (9.8)	230 (15.0)	51.6 (10.9)	120 (8)	440 (20)	−6.4 (0.8)	−4.2
	Old	404.6 (7.6)	150 (6.0)	67.5 (15.7)	130 (8)	375 (18)	−5.8 (0.8)	−2.0
2470	Young	549.1 (30.9)	410 (4.5)	176.3 (18.6)	75 (3)	620 (25)	−7.8 (0.6)	−8.3
	Old	523.7 (19.2)	400 (6.2)	127.7 (8.6)	70 (2)	602 (16)	−8.7 (0.3)	−7.5
Common garden:								
107	Young and old	333.8 (10.5)	207 (5.2)	13.2 (11.3)	116 (7)	495 (13)	−5.8 (0.4)	−5.6
1280	Young and old	416.0 (7.3)	262 (4.4)	28.7 (8.0)	106 (6)	465 (22)	−5.3 (0.8)	−6.2
2470	Young and old	458.6 (7.4)	350 (19.0)	72.2 (25.2)	95 (5)	620 (13)	−7.8 (0.3)	−6.8

Note. Values are means with standard errors in parentheses. Plants growing both on young (111–140-yr-old lava flows) and old (>2500-yr-old lava flows) substrates were studied for field plants, and the data for the common garden plants obtained from young and old substrate populations were pooled. LMA = leaf mass per area; LWC = leaf water content; LT_{50} = temperature when 50% of leaf tissue is damaged.

garden plant leaves followed the same pattern with elevation, but the ice nucleation temperature range was only 2°C. The average temperature at which 50% of the maximum irreversible cellular damage was observed (LT_{50}) for the lowest elevation field populations was ca. −1.9°C, whereas the LT_{50} for the high-elevation populations was substantially lower and was similar to its ice nucleation temperature. The LT_{50} of the common garden plants ranged from −5.6° to −6.8°C (table 1).

Leaf Characteristics, Osmotic Potential, and Water Content

The leaf water content of field plants decreased with increasing elevation (table 1). The decrease was not as large for common garden plants. The osmolarity of the leaf tissue was higher for the 2470-m-elevation population compared with the lower-elevation populations for both field and common garden plants (table 1). Lamina thickness and LMA increased with increasing elevation for both the field and common garden plants (table 1). Leaf mass per unit area increased threefold in the field plants, whereas it increased less than onefold in the common garden plants. This resulted from a decrease in leaf size rather than from an increase in leaf weight. Leaf weight remained essentially constant with increasing elevation (results not shown), but lamina thickness increased by only 70%, which indicates that the number of cells per unit volume of lamina must be greater at higher elevation. The amount of leaf pubescence also increased with increasing elevation for both common garden and field plants (table 1).

Ice nucleation temperatures and LT_{50} temperatures were positively correlated for both the field and common garden plants from different elevations; however, the slope of the linear relationship was steeper for the field compared with the common garden plants (fig. 6). The LT_{50} and the ice nucleation temperatures were similar for the high-elevation field and common garden plants. Ice nucleation temperature decreased 2°C as LMA per unit of lamina thickness increased from ca. 3.5 to 7.5 g/dm^3 (fig. 7). There was a smaller change in the LMA: lamina thickness ratio with respect to elevation in common garden compared with field plants. A single linear regression was fitted to both the field and common garden plants. Similarly, LT_{50} was also linearly related to changes in LMA per unit of lamina thickness for both field and common garden plants; LT_{50} decreased from ca. −2° to −8°C with increasing LMA/lamina thickness, and again, a single linear regression described the trends for both the field and common garden plants (fig. 7).

Nuclear Magnetic Resonance

The NMR studies were conducted with leaves obtained from field-grown plants from the high- and low-elevation sites. The NMR studies revealed that during freezing, a rapid drop in the leaf liquid water content occurred in the low-elevation leaves between −5° and −9°C (fig. 8). The liquid water content for the high-elevation leaves decreased during freezing at a slower rate. At −20°C, ca. 18% of the water remained in the liquid state in the low-elevation leaves, whereas the amount of liquid water remaining at −20°C in the high-elevation leaves was ca. 38%. The spin-spin and the spin-lattice relaxation rates of two water fractions in the leaf tissue determined at 0°C, before the onset of freezing, were higher in the high-elevation compared with the low-elevation plants (fig. 9).

Discussion

Air temperatures can fall below −5.3°C for meteorological stations above 2500 m elevation, the highest altitudinal distribution limit of *Metrosideros polymorpha* found anywhere in Hawaii (fig. 1). Large air-temperature gradients are usually found near the ground, particularly on cold nights. Thus, seedlings may experience even lower temperatures than those indicated by temperature sensors at a standard height above the

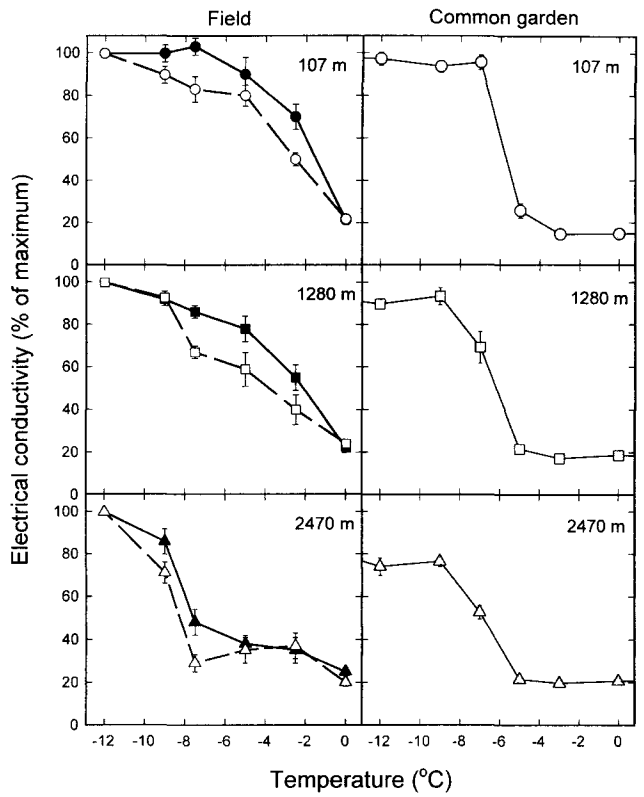

Fig. 5 Electrical conductivity (as percentage of maximum) of the bathing solution for *Metrosideros polymorpha* leaves as a function of temperature from field and common garden plants (seed source for the common garden plants was from the same field populations). Circles, squares, and triangles are from 107-m, 1280-m, and 2470-m elevations, respectively. Dashed lines and open symbols are from field plants growing on young substrates, and continuous lines and filled symbols are from field plants growing on old substrates. Shaded symbols are from common garden plants. Symbols are means ± standard error; $n = 5$ for field plants, and $n = 10$ for common garden plants.

ground. Noguchi et al. (1987) measured nighttime air temperature to surface temperature differences as great as 4.9°C near the summit of Haleakala volcano, at 3018 m above sea level, in Hawaii. Using temperature data from Pinadaude (3480 m above sea level on Mount Withelm, Papua, New Guinea), Noguchi et al. (1987) also showed that the difference between daily minimum air temperature and surface temperature increases as surface temperature decreases. Based on the Pinadaude data, surface temperatures as low as −8°C are possible at times when air temperatures at 1.5 m are still above 0°C. Our microclimatic data also showed that 4°–5°C differences between surface or near-surface air temperature and air temperature 1.5 m above ground are common. These various observations indicate that minimum air temperatures below −5°C, accompanied by surface temperatures that are 5°C colder, are possible at the elevation of the *M. polymorpha* tree line in Hawaii.

A large variation in leaf morphology and anatomy of *M. polymorpha* was observed along the altitudinal/temperature gradient. For example, LMA increases threefold with increasing elevation. This increase resulted from a decrease in leaf size rather than from an increase in leaf weight, which remained essentially constant along the altitudinal gradient. Despite the large increase in LMA, lamina thickness increased by only 70%, which indicates that other factors, such as the number of cells per unit volume of lamina, must have contributed to greater LMA at higher elevations. A higher degree of cell packing will result in lower intercellular spaces and therefore lower apoplastic water content, thereby increasing the supercooling capacity of the leaves (Goldstein et al. 1985). Small intercellular spaces may constrain extracellular ice formation or delay extracellular ice seeding, resulting in supercooling capacity enhancement. This hypothesis needs to be experimentally tested. The magnitude of freezing exotherms in *M. polymorpha* appeared to be related to the leaf water content. Both high-elevation field-grown and common garden plants developed small exotherms during tissue freezing and have relatively low water content, whereas low-elevation plants had large exotherms and relatively high water content. High supercooling capacity is associated with low apoplastic water content in giant rosette species in Andean tropical alpine plants (Goldstein et al. 1985). A strong relationship was found between relative apoplastic water content and the number of intercellular spaces observed in leaf sections of *Espeletia schultzii* giant rosettes (Rada et al. 1987). Leaves with small cell size, small intercellular spaces, the absence of ice nucleators, and relatively low tissue water content (Goldstein et al. 1985; Sakai and Larcher 1987) generally characterize plants that are able to supercool. Consistent with this trend, supercooling ca-

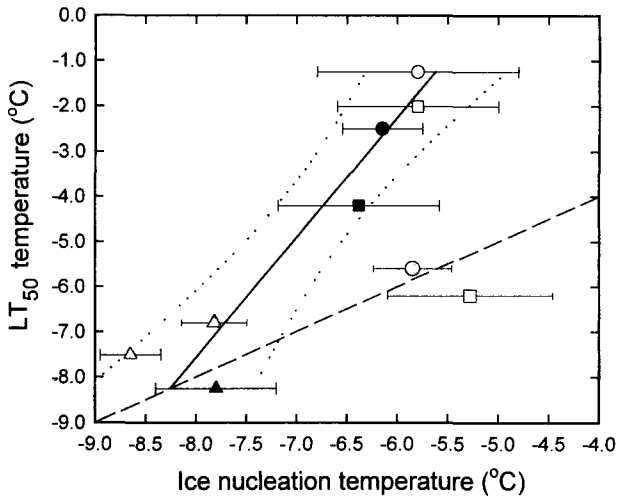

Fig. 6 Irreversible tissue damage temperatures (LT_{50}) versus ice nucleation temperatures for leaves of field and common garden *Metrosideros polymorpha* populations from 107-m (circles), 1280-m (squares), and 2470-m (triangles) elevations. Open symbols (young substrate age) and solid symbols (old substrate age) are from field plants. Shaded symbols are from common garden plants from both soil age substrates combined. The solid line represents the linear regression ($y = 0.38x - 5.15$, $r^2 = 0.88$) of field populations only, and the dotted line represents the 95% confidence interval. The dashed line represents the 1 : 1 relationship between ice nucleation and tissue damage temperatures. Values are means ± standard error; $n = 5$ for field plants, and $n = 10$ for common garden plants.

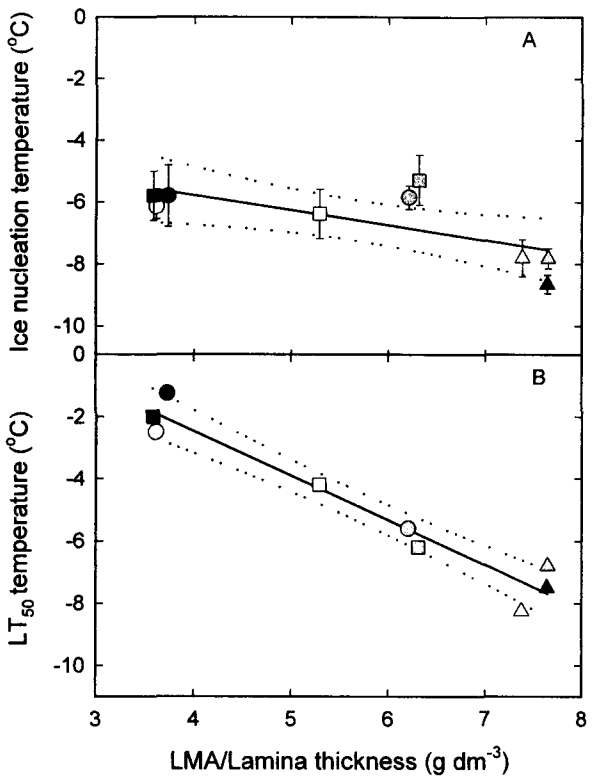

Fig. 7 Ice nucleation temperature (*A*) and 50% irreversible tissue damage temperature (LT_{50}; *B*) as a function of the ratio of leaf mass per area (LMA) and lamina thickness for field plants (open symbols are from young substrates, and solid symbols are from old substrates) and common garden plants (shaded symbols). The solid lines are the linear regressions fitted to all the data, and the dotted lines are the 95% confidence intervals in both panels *A* ($y = -0.48x - 3.88$, $r^2 = 0.50$) and *B* ($y = -1.43x + 3.23$, $r^2 = 0.94$). Symbols are means ± standard error in panel *A*; $n = 5$ for field plants, and $n = 10$ for common garden plants.

pacity in *M. polymorpha* leaves also appears to be enhanced by lower apoplastic water content, which is probably associated with small intercellular spaces and/or low cell wall surface area within leaves.

The amount of pubescence on *M. polymorpha* leaves increased significantly with elevation in field plants but not in common garden *M. polymorpha* plants. Pubescence accounted for up to 40% of the total leaf mass in the highest elevation field plants. The functional significance of leaf pubescence in *M. polymorpha* is not clear. It may play a role in increasing water use efficiency by reducing the rate of water loss (precipitation tends to decrease at high elevations). It can also play a role in freezing resistance by reducing the extent of leaf surface wettability and by therefore preventing direct contact between liquid water and the lamina of the leaves.

Under experimental conditions, leaves from low-elevation *M. polymorpha* populations exhibited substantial damage at temperatures close to −2°C. Typical of low-elevation tropical plants that are sensitive to low temperatures, chilling injury occurs at mild subzero temperatures before freezing occurs. However, leaves of high-elevation *M. polymorpha* field plants exhibited damage only at ca. −8.5°C, concurrent with ice formation in the leaf tissue, which is typical of plants that avoid freezing in their natural habitat by supercooling. Freezing damage to leaves was rarely observed in *M. polymorpha* high-elevation plants, despite the occurrence of subzero air temperatures at the upper limit of distribution of this species.

Spin-lattice and spin-spin relaxation rates (T_1^{-1} and T_2^{-1}, respectively) determined from NMR studies provide information regarding the state of water in cells and tissues (Goldstein and Nobel 1991; Veres et al. 1991), which may be useful information for understanding some characteristics of the freezing process. Spin-lattice relaxation rates provide a measure of the degree of water binding and/or viscosity, with higher T_1^{-1}'s resulting in stronger water binding to osmotically inactive structures such as cell walls, greater water viscosity as a result of solutes, or both. Spin-spin relaxation rates provide information on the mobility of water protons (Stout et al. 1978). The leaves of *M. polymorpha* trees from high-elevation sites had faster relaxation rates, which was consistent with their lower water content. It is possible that low apoplastic water content and a high degree of water binding in leaf tissues of *M. polymorpha* are prerequisites for the enhancement of permanent supercooling (*sensu* Larcher 1973).

Nuclear magnetic resonance studies with alpine Hawaiian plants revealed that a rapid drop in the leaf liquid water content occurred during freezing in species that avoid freezing by supercooling (Lipp et al. 1994). The liquid water content of freezing tolerant plants, however, tended to decline at a substantially lower rate during freezing, probably as a result of intracellular water being distilled slowly to the ice nuclei located in the apoplastic spaces of the leaves. When freezing is experimentally induced in *M. polymorpha* leaves from low-elevation populations, the rate of decline of liquid water is fast and is similar to that of *Dubautia menziesii*, a Hawaiian alpine plant that avoids freezing by supercooling (Lipp et al. 1994). The rate of decline of liquid water during freezing in leaves

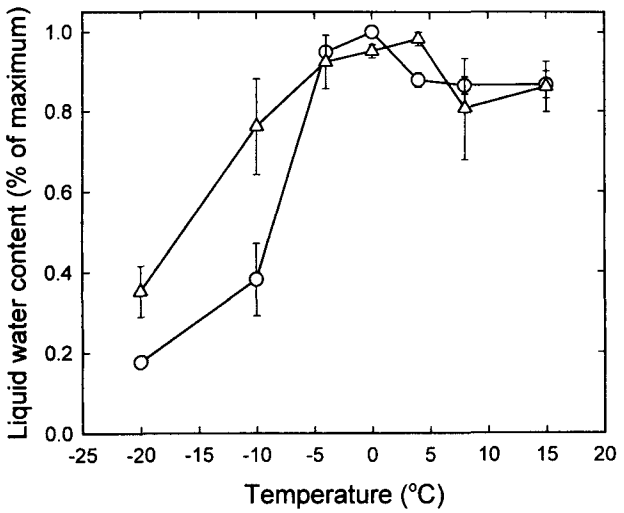

Fig. 8 Liquid water content determined by nuclear magnetic resonance of low- (circle) and high- (triangle) elevation leaves from *Metrosideros polymorpha* field plants. Symbols are means ± standard error; $n = 3$.

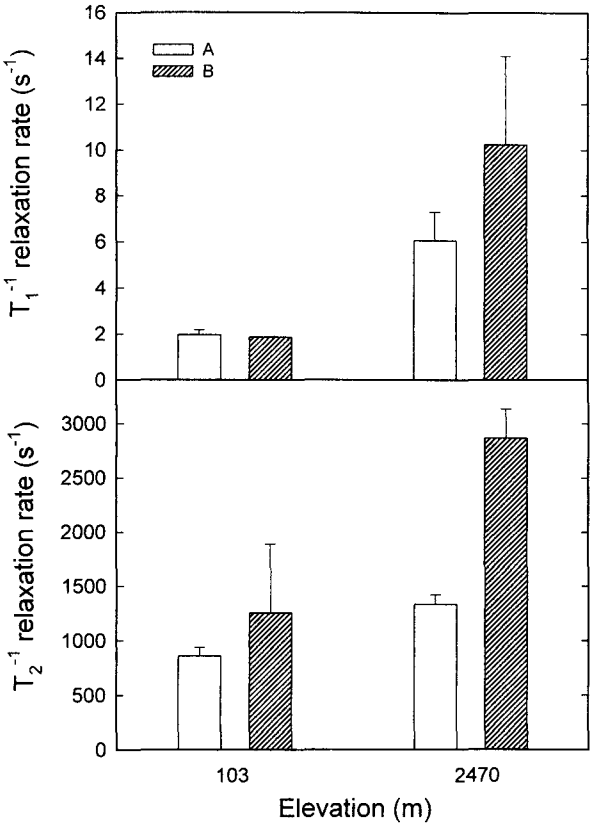

Fig. 9 The spin-lattice (T_1^{-1}) and spin-spin (T_2^{-1}) relaxation rates determined at 0°C of low- and high-elevation leaves from *Metrosideros polymorpha* field plants. Open bars represent spectrum *A*, presumably intracellular water, and crosshatched bars represent spectrum *B*, presumably extracellular water. The bars are means + standard error; $n = 3$.

from high-elevation *M. polymorpha* plants is slower and partially resembles the pattern of liquid water decline during freezing observed in the more freezing-tolerant alpine Hawaiian plants. Ice formation tends to occur close to the equilibrium freezing temperatures in freezing-tolerant plants. It is believed that the advantages, for freezing-tolerant plants, of initiating freezing closer to the equilibrium freezing temperatures (and therefore of reducing the extent of supercooling) are to avoid cellular damage and to reduce the magnitude of initial cellular dehydration resulting from the movement of symplastic cellular water to the growing extracellular ice crystal (Krog et al. 1979; Beck et al. 1982). It is possible that despite the larger supercooling capacity of the high-elevation *M. polymorpha* leaves, intracellular ice formation is delayed after ice seeding in the extracellular leaf spaces. It appears that high-elevation *M. polymorpha* have an incomplete suite of leaf characteristics, which would lead to either a strict avoidance or strict tolerance of extracellular ice formation, perhaps as a result of the recent evolutionary status of this species in the isolated Hawaiian archipelago.

Metrosideros polymorpha is extremely variable in morphology and physiology. A recent study has shown that certain physiological and anatomical traits are plastic and are largely induced by the environment, whereas other characteristics, particularly leaf morphology, are fixed and are determined by genetic background (Cordell et al. 1998). The results from our current field and common garden study indicate that the enhanced supercooling capacity and the lower freezing damage temperatures of high-elevation plants were a result of a combination of environmentally induced and genetically based traits. Comparison of leaf traits between field- and common garden–grown plants indicated that the range of variation was larger for field-grown plants. For example, the average difference in LMA/lamina thickness between the lowest and highest elevation leaves was 2 g/dm^3 for common garden plants, compared with 5 g/dm^3 for field plants. The ice nucleation temperature range was only 2°C for the common garden populations, as opposed to 3°C for the field populations. The range of freezing damage was ca. 1°C for common garden plants, compared with a range of >6°C for field plants. However, the ice nucleation and LT_{50} temperatures as well as LMA/lamina thickness of field and common garden high-elevation plants were very similar, whereas the same traits in low-elevation field and common garden plants were substantially different. It appears that *M. polymorpha* plants growing at high elevation have been under strong selective pressures for the evolution of freezing-resistance mechanisms. As a result of this selective pressure, some of the anatomical and morphological characteristics of leaves have been genetically fixed, in particular, high LMA and cell density and small extracellular spaces for ice seeding, traits that apparently enhance the supercooling ability of high-elevation *M. polymorpha* plants.

In a recent reassessment of global climate-driven tree line, Körner (1998) suggested that root zone temperature of emergent seedlings in the shade of large adults is likely to be the most critical factor on climate-driven tree line formation and that growth limitations are more important limitations than are CO_2 assimilation and carbon balance. Körner found no correlation between annual absolute minima of air temperature and tree line position and concluded that frost damage is unlikely to play a decisive role in tree line formation on a global scale. On the other hand, it has been suggested that deep supercooling (supercooling to temperatures near −40°C) of xylem tissue in timberline tree species of the Colorado Rocky Mountains may be a factor limiting survival of trees at high elevation (Becwar et al. 1981). In the above study, however, extracellular ice formation in the stem samples first occurred at substantially higher subzero temperatures (−10° to −13°C), but no damage was observed until temperatures approached −40°C. Our data indicate that the supercooling capacity of *M. polymorpha* leaves at high elevation is only large enough to allow avoidance of leaf tissue damage as a result of freezing up to 2500 m elevation, where *M. polymorpha* forms tree line on Mauna Loa. If this is confirmed for the few other high-elevation species, such as *Sophora crysophylla*, it will be the first example to show that freezing damage resulting from limited supercooling capacity of foliage can be a factor in tree line formation.

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