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ORIGINAL RESEARCH

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Infrared thermography of in situ natural freezing and mechanism of winter-thermonasty in *Rhododendron maximum*

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Abstract

Evergreen leaves of *Rhododendron* species inhabiting temperate/montane climates are typically exposed to both high radiation and freezing temperatures during winter when photosynthetic biochemistry is severely inhibited. Cold-induced "thermonasty," that is, lamina rolling and petiole curling, can reduce the amount of leaf area exposed to solar radiation and has been associated with photoprotection in overwintering rhododendrons. The present study was conducted on natural, mature plantings of a cold-hardy and large-leaved thermonastic North American species (*Rhododendron maximum*) during winter freezes. Infrared thermography was used to determine initial sites of ice formation, patterns of ice propagation, and dynamics of the freezing process in leaves to understand the temporal and mechanistic relationship between freezing and thermonasty. Results indicated that ice formation in whole plants is initiated in the stem, predominantly in the upper portions, and propagates in both directions from the original site. Ice formation in leaves initially occurred in the vascular tissue of the midrib and then propagated into other portions of the vascular system/venation. Ice was never observed to initiate or propagate into palisade, spongy mesophyll, or epidermal tissues. These observations, together with the leaf- and petiole-histology, and a simulation of the rolling effect of dehydrated leaves using a cellulose-based, paper-bilayer system, suggest that thermonasty occurs due to anisotropic contraction of cell wall cellulose fibers of adaxial versus abaxial surface as the cells lose water to ice present in vascular tissues.

1 | INTRODUCTION

Rhododendron is a wide-spread woody perennial genus of *Ericaceae* with >1000 species, many of which occur in the temperate zone and montane habitats where they must survive extended periods of sub-freezing temperatures in winter. They do so primarily by undergoing cold acclimation and increasing their freezing tolerance during fall and winter. A major component of plant cold hardiness, including rhododendrons, is their ability to tolerate "freeze-dehydration" due to the loss of osmotically active cellular water to extracellular ice in response to a vapor pressure gradient (Hansen & Beck, 1988). Extracellular ice formation occurs as a consequence of ice-nucleation (Arora, 2018; Wisniewski et al., 2018). Therefore, determining sites of ice-nucleation, its progression, and accommodation within the plant is crucial for understanding the ability of a plant to tolerate extracellular ice and its related stresses.

To the best of our knowledge, three studies have been conducted on ice formation and propagation in rhododendrons, two of which employed temperature-controlled laboratory protocols to freeze excised leaves/leaf whorl of relatively large-leaved north American species (*Rhododendron catawbiense* and *Rhododendron* sp. cv. Olga) (Arora et al., 2021; Wisniewski et al., 1997), whereas one study involved in situ freezing of a relatively small-leaved, dwarf rhododendron shrub (*Rhododendron ferrugineum*) from Alpine timberline (Hacker & Neuner, 2008). No studies have been conducted on freezing response (preferred sites of ice-nucleation and propagation) in

field-established, large-leaved rhododendrons under natural freezing events.

Many *Rhododendron* species are broad-leaf plants that are typically exposed to both high radiation and freezing temperatures simultaneously during winter when photosynthetic electron consuming assimilation reactions are inhibited. This represents a combination of cold and light stresses for which overwintering rhododendrons must employ strategies to manage excess excitation energy from photon overload to prevent photo-oxidative damage (Nilsen et al., 2014; Wei et al., 2005). Rhododendrons have evolved mechanisms to dissipate excess absorbed light as thermal energy (nonphotochemical quenching) via the xanthophyll cycle, protect against photooxidative damage via antioxidants, accumulate protective proteins, such as early light-induced proteins, reduce the amount of light harvesting by decreasing leaf chlorophyll, and/or undergo adjustments in photosynthetic and secondary metabolism (Harris et al., 2006; Liu et al., 2019, 2020; Peng et al., 2008; Wang et al., 2008, 2009; Wei et al., 2005).

Cold-induced leaf movement, termed "thermonasty," can reduce the amount of leaf area exposed to solar radiation and has been associated with photoprotection in some overwintering rhododendrons (Bao & Nilsen, 1988; Russell et al., 2009); thermonasty in rhododendrons includes both petiole curling (change in leaf angle) and inward rolling of the leaf lamina, thus enclosing the abaxial surface (Arora et al., 2021; Nilsen, 1992). Nilsen et al. have extensively studied the eco-physiological aspects of thermonasty in rhododendrons (Nilsen et al., 2014 and references therein), but the presumptive relationship of leaf curling and rolling in nature with confirmation of in situ ice-formation in tissues has not been documented.

The present study was conducted on natural, mature plantings of a cold-hardy and a large-leaved thermonastic North American *Rhododendron* species (*Rhododendron maximum*) during winter-freezes. Infrared thermography was used to address the following questions: (1) where in the plant and at what subfreezing temperature is ice formation initiated at the whole plant level, and what are the dynamics of ice propagation?; (2) what is the temporal relationship between ice formation (ice-nucleation) and leaf thermonasty, that is, which precedes the other, or do they occur simultaneously?; and (3) what are the primary sites of ice accommodation in the leaves? The histology of winter leaves and the information derived on ice-formation, propagation, and accommodation inside the leaf, together with a simulation of the rolling effect of dehydrated leaves using a cellulose-based, paper-bilayer system, were used to elucidate the putative spatial relationship between ice-formation and thermonastic leaf movements and propose a mechanism for winter thermonasty in *Rhododendron*.

2 | MATERIALS AND METHODS

2.1 | Plant material

Mature *Rhododendron maximum* L. plants living under natural conditions adjacent to the Pisgah National Forest in Montreat, North Carolina (35° 38′ 23.4″ N, 82° 18′ 26.6″ W), at an altitude of 803 m were evaluated for freezing patterns and subsequent thermonasty of leaves during the winter of 2021–2022. A 1 × 2 m^2 section of the forest at the location was cleared of plant litter (Figure S1), and the leaves and stems of several approximately 3–6 m tall shrubs with 5–10 cm diameter stems/trunks were selected for monitoring with two infrared cameras. Freeze events at the location were recorded during the night on November 19 and 20, 2021, December 19, 2021, and January 10, 2022.

2.2 | Infrared thermography

A FLIR T620 infrared camera (640 × 480 resolution) with an $f = 24.6$ mm lens was used to capture freeze events at the soil surface (Figure S2). A FLIR A8583 (1280 × 1040 resolution) with a 50-mm lens, interfaced by an Ethernet cable to a laptop computer was used to image leaves and trunks and a 3×, F2.5 microscope lens was used to image cut surfaces of leaves and petioles.

Continuous video recordings were captured on screen at 30 frames per second using the BandiCam software. IR monitoring of freezing began when the air temperature reached 0°C (around 9:00 p.m.) and ended around 7:00 a.m. the next morning when air temperatures were around −7 to −9°C. After recording, screen-captured MP4 files were converted to a .mov format and edited for presentation using Adobe After Effects.

2.2.1 | Microscopic IR imaging (petiole, leaf)

After cutting away a leaf lamina, the petiole that remained attached to the stem was cut longitudinally with a razor to film internal freezing. Leaves still attached to stems were cut in cross sections and oriented so that the cut surface was flat to the 3× microscope lens.

2.3 | Histology

Rhododendron mediolateral leaf segments with midribs and leaf base petioles (including some leaf tissues) were fixed in modified FAA (methanol was substituted for EtOH) for 2 h at 30°C and then incubated in 70% EtOH overnight in 58°C water bath. A 5% graded ethanol series from 70 to 100% was used to dehydrate the samples for 1 h in each solution at 58°C. After dehydration, samples were moved into a 1:1 EtOH:2-propanol, followed by two changes of 100% 2-propanol and two changes of 1:1 2-propanol: paraffin for 30 min at each step. Samples were then transferred to paraffin for infiltration for 48 h with four changes of fresh paraffin (the first two changes were after 4 h each, and the subsequent changes were after overnight incubation). After blocking, they were sectioned at 20 microns, placed on microscope slides, de-paraffined with xylene, stained with a Saffranin, and Fast Green double stain, covered and then photographed with a Cannon Rebel T3i camera mounted on a Nikon, Eclipse 50i microscope at 100 and 200×.

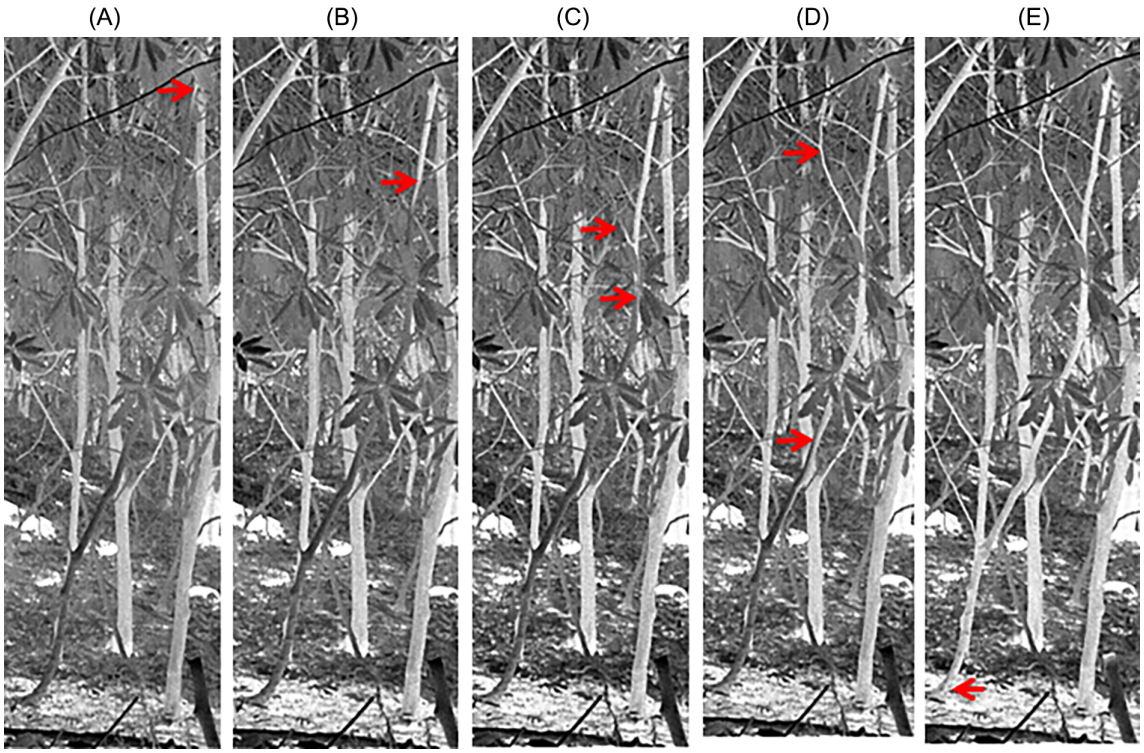

FIGURE 1 Freeze frames from an infrared video of *Rhododendron maximum* freezing from the top of the trunk to the bottom. Red arrows indicate the progression of freezing. Note that in (C) freezing moves into a branch and proceeds upwards as well as down. Red arrow in (E) shows freezing that had progressed to the base of the tree. The progression of freezing is much easier to follow in Video S2.

2.4 | Paper bilayer

A paper bilayer was created by cutting two 2 × 12 cm^2 strips in two directions, with and against the lines from a lined, yellow Ampad (Tops-products.com). The strip cut *with* the lines expanded from 12 to 12.3 cm after 10 min when placed in water, while the strip cut *against* the lines did not expand along its 12 cm length when wet. The expanded strip was sandwiched to the nonexpanded strip with a thin layer of silicon caulk and allowed to dry. This created an anisotropically arranged bilayer with two separate coefficients of moisture expansion (CME) (Schroeder, 1972).

3 | RESULTS AND DISCUSSION

3.1 | General freezing patterns

3.1.1 | Soil

The soil surface was the first freeze event observed in all infrared thermographic observations (Figure S2) and did not collectively freeze at the same air temperature but rather froze in patches at varying temperatures from just below freezing to around −3°C (Video S1). Arrows point to two unfrozen patches (Figure S2A) in the midst of larger areas that had frozen previously; the lighter shade of the same areas in Figure S2B indicates that freezing had occurred. When the air temperature reached about −3°C, the entire visible soil surface was seemingly completely frozen. Importantly, however, the depth of ice formation was not determined. The freezing of soil in patches under natural conditions has been previously reported (Livingston III et al., 2018). Differences and interactions between soil moisture, the presence and quantity of diverse nucleating agents, and/or variations in the microclimate likely contributed to the segmented freezing pattern.

3.1.2 | Stems/trunks

As the soil froze, ice formation was initiated at different locations of the stems/trunks of the rhododendron shrubs (Figure 1) (refer to the video recording [Video S2] for a distinct freezing dynamic). Freezing in trunks occurred at varying temperatures during the night ranging from −2 to −7°C (Video S2). Generally, in freezing studies, thermocouples are used to detect an exotherm and ascertain ice formation in plant tissues. Our observations highlight the limitation of using thermocouples to determine a precise ice nucleation temperature when studying the freezing behavior of whole plants under natural conditions, such as "freezing point of which tree"? or "which tissue of which tree"? Because energy in the form of heat is released during freezing and infrared thermography can localize the initiation of freezing to specific regions of plants, this technology has been referred to as "tissue-specific calorimetry" (Livingston III et al., 2018). Others have used

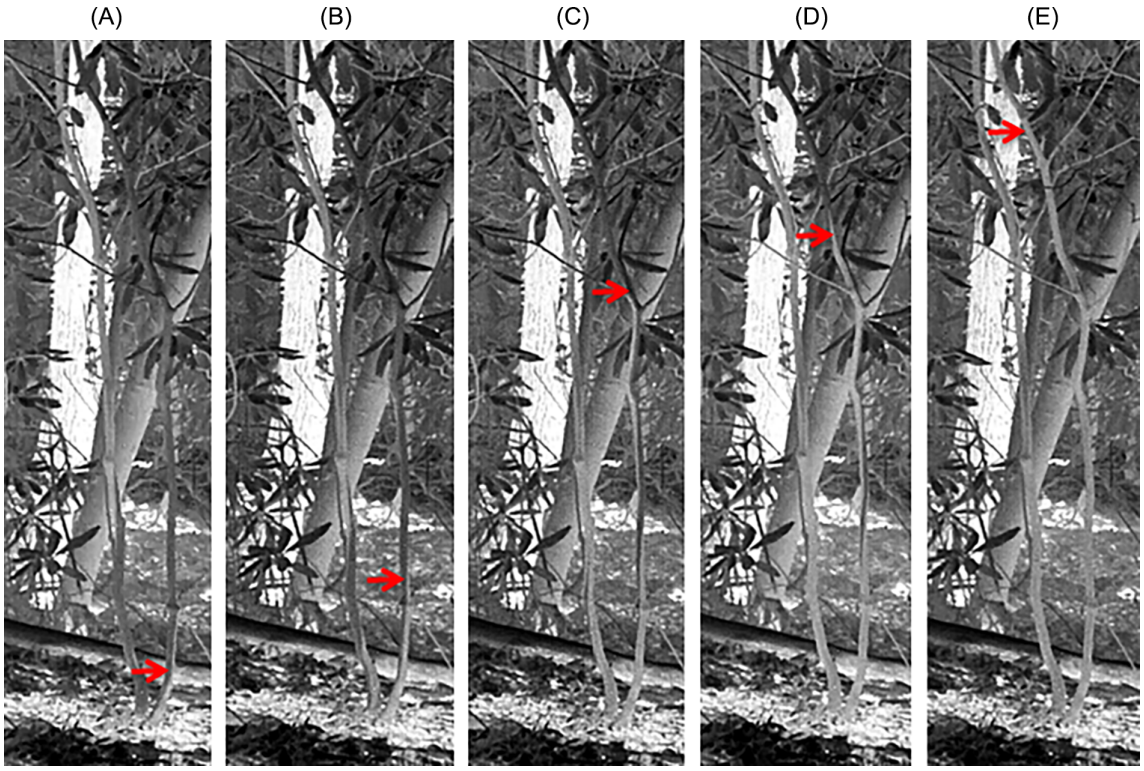

FIGURE 2 Freeze frames from an infrared video of *Rhododendron maximum* freezing from the base of trunk to the top. The progression of freezing is much easier to follow in Video S2.

infrared thermography to study freezing in various plant models (Fuller et al., 2003; Kuprian et al., 2014; Workmaster et al., 1999).

In most cases freezing began in the upper regions of trunks (Figure 1A–E and Video S2) and moved downward, but in some cases, freezing began at the bottom and progressed upward (Figure 2A–E and Video S2). A similar spatially erratic pattern of ice progression upon freezing (apex to bottom or vice-versa) has been observed in the branches of apple and *Picea abies* trees (Charrier et al., 2017; Pramsohler et al., 2012). Conceivably, the site of initial freezing is related to the probability of ice-nucleation, a stochastic process that depends on the temperature, among other factors. Pramsohler et al. (2012) argued that the direction of ice progression in apples depended on soil temperature and observed that ice initiated at the base of apple trees and propagated upward when the soil was frozen, whereas the ice wave started in upper stems and moved downwards if the soil was not frozen.

Previous IR recordings of freezing in wheat (Livingston III et al., 2016, 2018, 2021) under natural as well as controlled conditions indicated that freezing always began at the base of the plant and proceeded upward. This was even the case when wheat leaves were removed and plants were frozen with cut ends exposed. It has been suggested that freezing is initiated in leaves at wound sites, hydathodes or stomates (Wisniewski et al., 2014). However, this pattern was not observed with rhododendrons, wherein freezing began in trunks and stems instead of leaves (Video S3). Also, it did not initiate at wound sites, neither in apparently cured, preexisting wound (Figure 3A; upper wound) nor in a freshly wounded site (Figure 3A, lower wound) (Video S3). Our results support earlier observations by Hacker and Neuner (2008) that once initiated in the branches of *R. ferrugineum*, ice spread into the leaves, typically via venation.

3.1.3 | Leaves

Freezing usually began near the upper region of the trunk and spread into leaves, but it never appeared to begin in leaves (Figure 4 and Video S4). Freezing at the top of the trunk originated at an unknown location in the stem and then spread acropetally through the vascular bundles of the petiole (Figure 5 and Video S5) and into the vascular system of the leaves. Interestingly, freezing was localized in the vascular system and was never observed outside the vascular bundles, either in the petiole (Figure 5 and Video S5) or in the leaf (Figure 6 and Video S6). Wheat, oats (Livingston III et al., 2018), barley, rye, and other plant species (Hacker & Neuner, 2008; Stier et al., 2003) were also found to freeze at the base of the plant and spread into the leaves through the vascular bundles. After this initial freeze event in small grains, a second more ubiquitous and brighter (more heat released) exotherm spread throughout the leaf. This second exotherm in wheat occurred over a longer period of time and also took longer to come to equilibrium with its surroundings, presumably because it involved more water freezing. Initial freezing in the vascular system is called "stage one" freezing, and subsequent freezing encompassing the entire leaf "stage two" freezing (Livingston III et al., 2018, 2021); stage two freezing is presumably a result of free water freezing in the apoplast of the leaf. Unlike freezing in small grains, stage two freezing

FIGURE 3 Freeze frames of a portion of the trunk of a *Rhododendron maximum* tree. The diameter of this part of the trunk is 3.1 cm diameter and the 2 scars are 1.7 m from the soil surface. (A) Visible image of the two scars. The topmost scar is at least 1 year old and shows prominent healing at the edges. The bottom scar was made the same day as the recording of the freeze. (B) the same area as (A) prior to freezing. (C) Freezing beginning to move downward and along the side of the mature scar. (D,E) Freezing beginning at the side of the fresh scar and moving both vertically and horizontally across the scar. (F) Freezing covering both scars. Note that freezing was not initiated at either of the scars. The progression of freezing is much easier to follow in Video S3.

was not observed in *R. maximum*. Therefore, it is proposed that "stage three" freezing (freeze-dehydration), as suggested by Livingston III et al. (2018) ensued as the temperature descended below the freezing point of the vascular system. This presumably entails the efflux of cellular water from the leaf mesophyll to the site of ice in vascular bundles due to a vapor pressure/water potential gradient (Arora, 2018).

3.2 | Thermonasty

3.2.1 | Leaves and petiole

Freezing in leaves began when air temperatures were between −2 and −4°C. Once the vascular system had frozen and the leaves returned to temperature equilibrium, no additional exotherm was observed (not shown). However, at one or two degrees colder than the freeze-initiation temperature, leaves began to roll inward, with the mid-rib serving as the central axis (Figure 4G,H and Video S4). Shortly after the start of leaf-rolling, the petiole of each leaf began to curl downward markedly beyond the initial, modest curling that typically occurs during cold acclimation (Arora et al., 2021). The entire leaf then became oriented in a position that was more vertical than it was prior to freezing (Figure 4H). The extent of rolling/curling continued to increase as air temperatures decreased, confirming previous findings (Nilsen, 1987). The following morning when the air temperature increased above 0°C and the leaves thawed, they unrolled to their original flat shape, and petioles straightened, allowing leaves to regain their pre-frozen configuration (not shown).

These cold-induced leaf movements, rolling and curling, constitute thermonasty in broadleaf evergreen rhododendrons (Nilsen et al., 2014). Early studies of thermonasty in rhododendrons were first

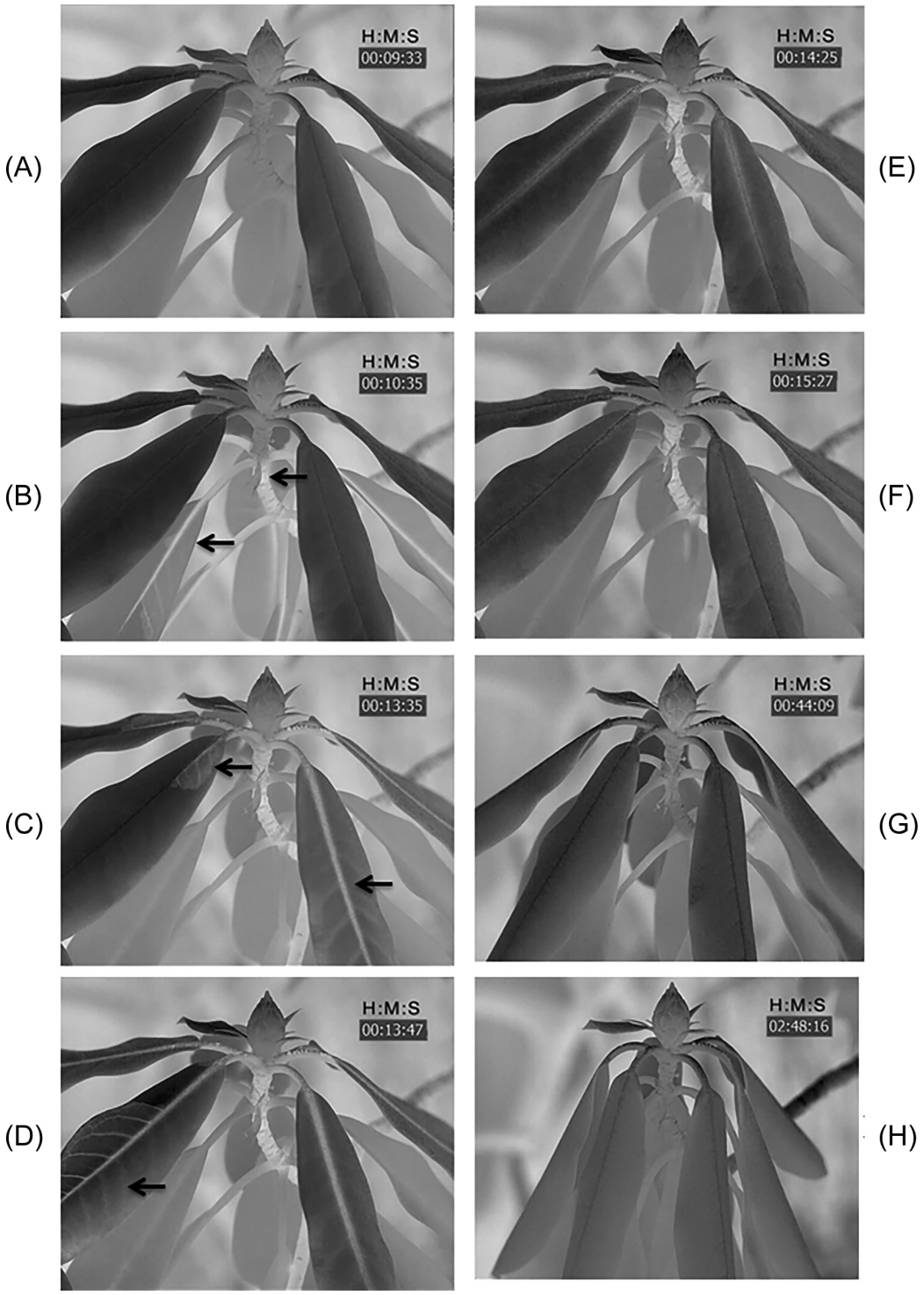

FIGURE 4 Infrared images of freezing in *Rhododendron maximum* leaves. (A) Top of a branch prior to freezing. (B) Arrows show freezing in the stem and a leaf in the whorl one layer beneath the topmost whorl of the branch. (C) Arrows show freezing following the pattern of the vascular system beginning in the uppermost whorl of leaves. (D,E) Freezing completed in the leaves of the uppermost whorl. (F–H) Thermonastic rolling of the leaves and curling of the petiole *after* all visible freezing events have occurred. Freezing was initiated at −3°C (B) and maximum thermonastic positioning (H) occurred nearly 3 h later at −5°C. The progression of freezing in leaves as well as the thermonastic movements are much easier to follow in Video S4.

conducted in the late 1800s (Harshberger, 1899), although Hooker (1849) first reported these leaf movements in rhododendron during Himalayan expeditions. It has been suggested that thermonasty could serve as a photoprotective strategy in overwintering rhododendrons, which harvest more radiation than their capacity to utilize it due to sluggish photosynthetic biochemistry under freezing temperatures.

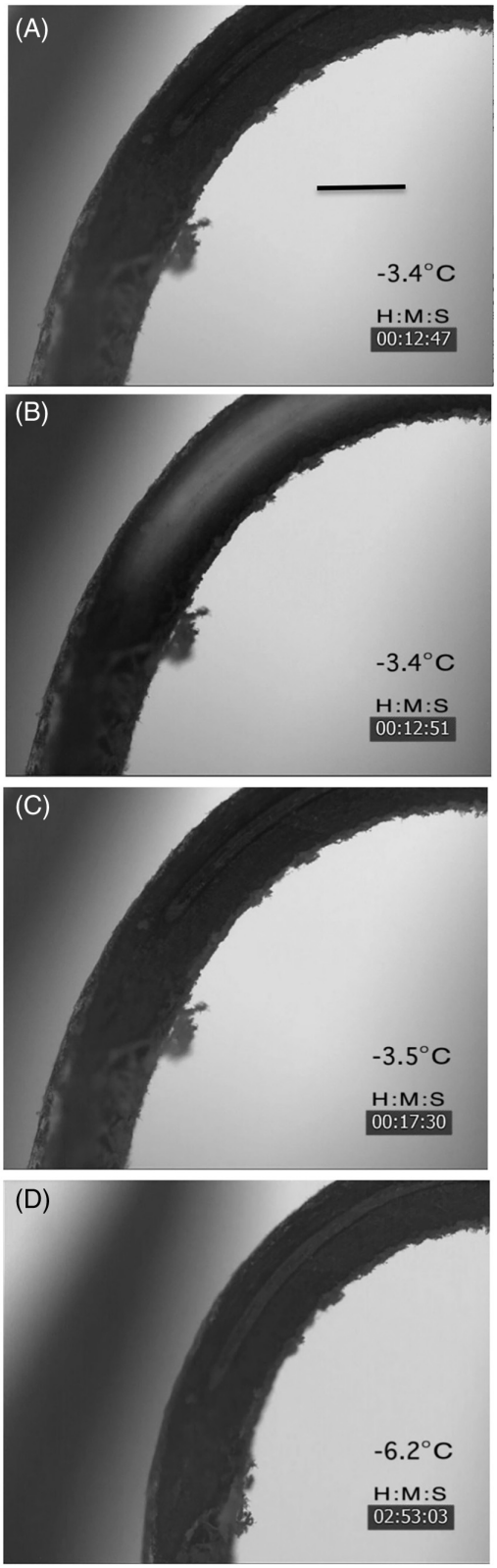

FIGURE 5 Infrared image of an *Rhododendron maximum* petiole that had been cut longitudinally to expose the interior tissues. Lighter shaded portion of the interior of the petiole in (B) indicating freezing where the vascular bundle is located. (C) Freezing of the vascular bundle completed and curling of the petiole (D) after the vascular system froze and came to equilibrium. Bar represents 1 mm. The progression of freezing as well as the petiole curling is much easier to follow in Video S5.

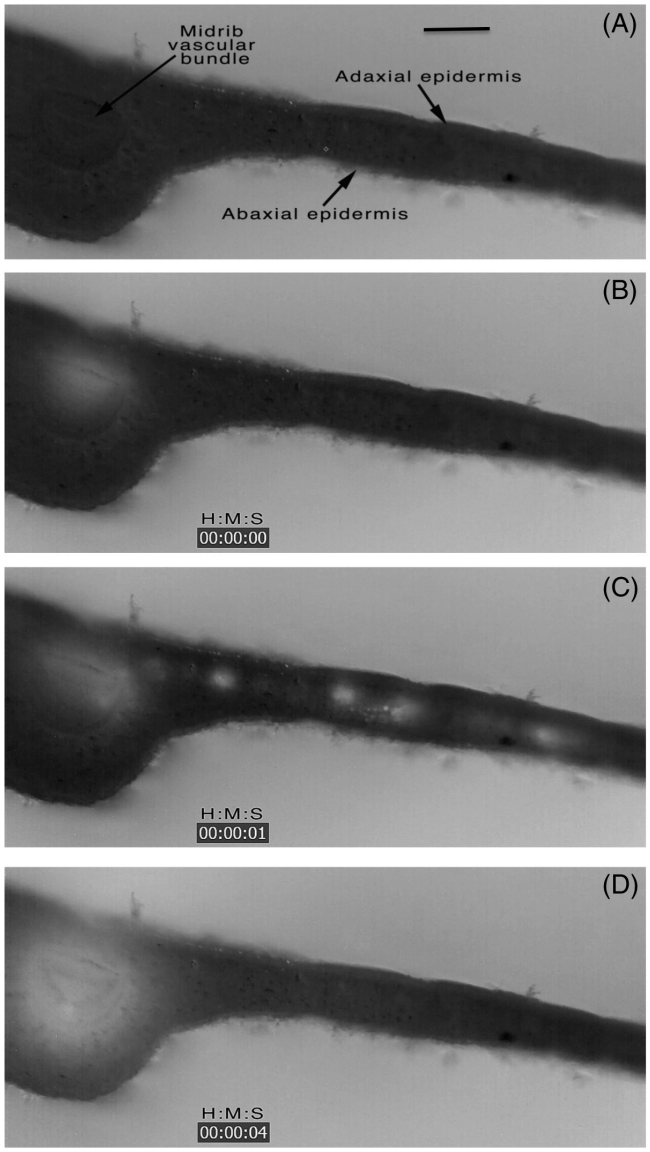

FIGURE 6 Infrared image of an *Rhododendron maximum* leaf that had been cut in cross section to expose the interior tissues. (B) Freezing beginning at the edge of the midrib. (C) Freezing confined to branches of the vascular system in the margin of the leaf lamina. Bar represents 1 mm. The progression of freezing is much easier to follow in Video S6.

Surplus excitation energy can potentially cause photo-damage, whereas thermonasty would reduce the absorbance of excess light (Arora et al., 2021; Bao & Nilsen, 1988; Russell et al., 2009; Wang et al., 2009; Wei et al., 2005). Additionally, desiccation-avoidance, reduced heat load, protection from mechanical damage due to the weight of ice/snow, and avoidance of potential rapid-thaw induced damage by slowing the thaw rate have also been proposed to be of adaptive significances of thermonasty during freezing cold (Nilsen, 1992).

Our observations under natural conditions demonstrated, on four different occasions, that leaf rolling initiated, and petiole curling intensified after ice formed in the vascular system, while no freezing was

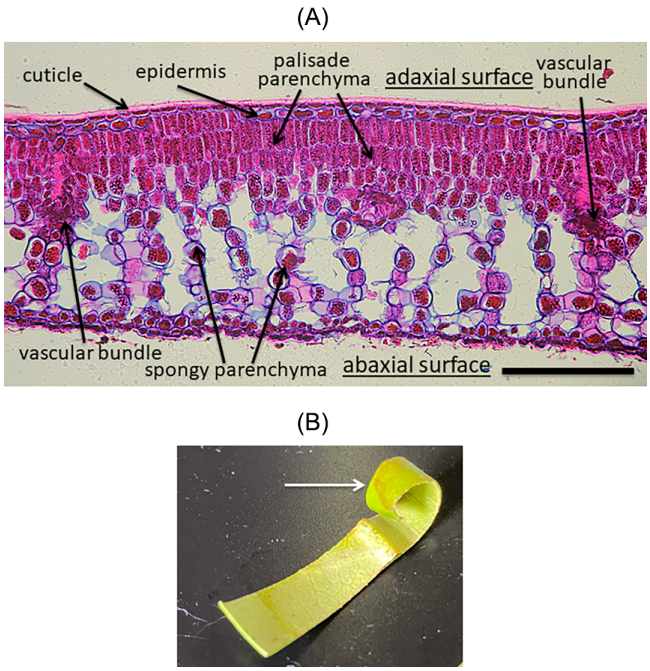

FIGURE 7 (A) Light microscope image of a cross-section of *Rhododendron maximum* leaf at 200×. Note air spaces between spongy mesophyll cells. The bar is 100 microns. (B) A 2-cm wide cross-sectional slice of an *R. maximum* leaf with the abaxial surface shown. The abaxial epidermis and a portion of the spongy parenchyma were removed with a scalpel from the right half of the leaf section (arrow) under a dissecting microscope. Note that the intact half of the leaf remains relatively flat while the half from which the lower epidermis was removed (arrow) had rolled considerably as it dried within approximately 2 h.

observed in either the epidermis, palisade, or spongy parenchyma cells (Figures 5 and 6 and Videos S5 and S6). In fact, rolling/curling continued to occur for several hours with no additional visible exotherm. This observation is in contrast to the suggestion that *Rhododendron* leaves, including *R. maximum*, begin to roll prior to freezing (Nilsen, 1991). Results from earlier studies with excised petiolated leaves of *R. catawbiense* are congruent with our observations that leaf freezing (ice-nucleation) indeed precedes thermonasty, especially the lamina-rolling component (Arora et al., 2021; Chen et al., 2013).

Because a visible exotherm did not occur in the epidermis, palisade, or spongy parenchyma during leaf and petiole movements, it may be concluded that thermonasty was not caused by differential ice formation between adaxial and abaxial layers, as suggested by Nilsen (1987), but was primarily a result of differential freeze-desiccation (more in the following sections). As early as 1899 Harshberger (1899) suggested that thermonasty in *R. maximum* resulted from the movement of "liquid" from cell to cell "so that one part of the leaf becomes turgescent and the other part more or less flaccid". Nilsen (1987) reported that changes in leaf turgor potential were related to petiole curling but not lamina rolling.

The vapor pressure of supercooled water is higher than that of ice at the same temperature (Salt, 1963). In fact, the lower the temperature, the greater the vapor pressure difference, at least until approximately −12°C (Salt, 1963). The vapor pressure difference between the vascular system (containing ice) and the mesophyll (palisade, spongy parenchyma) infers that a chemical potential gradient exists from turgid cells to the frozen vascular bundles. This suggests that water vapor moves from the palisade and/or spongy parenchyma to the vascular bundles. The cells closest to the vascular bundles and with the least anatomical restriction to the movement of water vapor would be the most likely to dehydrate, at least initially. This would be the spongy parenchyma (Figure 7A) on the abaxial side of the leaf as well as the layer(s) of palisade parenchyma on the adaxial side proximal to the vascular system. If the upper epidermal layers furthest away from the vascular bundles maintain their turgidity, then the overall effect would result in the rolling of the leaf. Cryo-SEM images of frozen leaves of *Trifolium repens* and *Escholschzia californica* demonstrate water movement out of parenchyma tissues to ice formed outside the cell surfaces, causing them to shrink (McCully et al., 2004). These authors demonstrate that when thawed, the water moved back into cells causing them to regain their original turgor. Cryo-SEM was not performed in the present study; therefore, our interpretation of the site of ice formation is mainly based on the visualization of exotherms that were only apparent in the vascular system. Accordingly, ice formation does not seem random but localized to specific areas of the leaves, suggesting that the leaf mesophyll and vascular tissues in this species presumably evolved specific cell wall properties that allowed for tissue separation to accommodate ice formation.

3.3 | Dehydration to explain thermonasty

3.3.1 | Leaves

In addition to leaf movements resulting from freezing temperatures, *Rhododendron* leaves, as well as other species, also roll when drought-stressed, or when they die (Hsiao et al., 1984; Nilsen, 1987; Satter & Galston, 1981; Zulfiqar et al., 2022). When an *R. maximum* leaf was killed by placing it in an oven at 60°C overnight, it rolled like it did when it was frozen (Figure 8A). After subsequently submerging the leaf in water, it completely unrolled (Figure 8B). This suggests that this nastic movement is a mechanical effect caused by the removal of water; in fact, it was termed "hygronasty" by Fukuda (1932). The same nastic effect was observed when *R. maximum* leaves were removed from trees, allowed to dry, and then placed in water to rehydrate them (not shown). Fukuda suggested that in the case of a dead leaf, rolling was caused by the contraction of the cell membrane by the depletion of water and that subsequent unrolling was due to water adsorption by cell membranes. While agreeing with this notion, we suggest further that the rolling and unrolling of a dead leaf in response to removal and addition of water demonstrates an anisotropic response to dehydration, that is, unequal degree and/or direction of shrinkage by the abaxial and adaxial surfaces and/or between layers of cells within leaves.

Fukuda (1932) also speculated that contraction of the "framework" of the spongy parenchyma on the lower side of the leaf during desiccation would enable the entire leaf to roll. However, when the

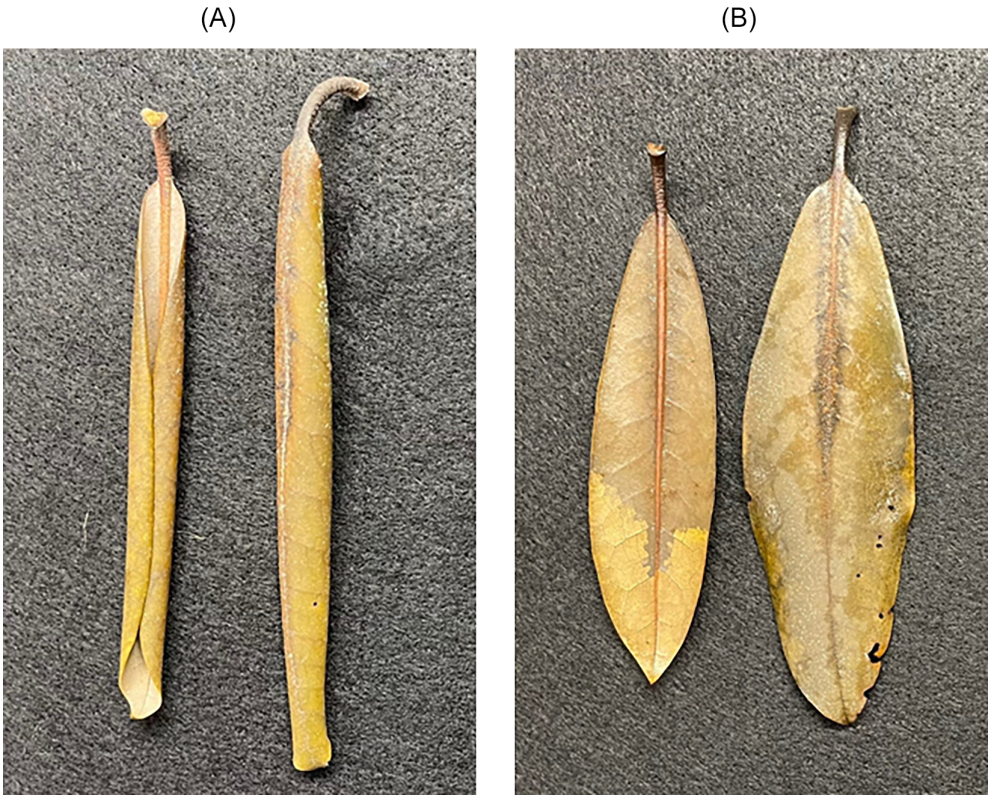

FIGURE 8 (A) *Rhododendron maximum* leaves dried in oven at 60°C overnight. (B) The same two leaves after being killed in oven and left in water for 7 days.

lower epidermis and a portion of the spongy parenchyma was removed from one-half of a 2-cm transverse section of a leaf using a scalpel under a dissecting microscope, the remaining adaxial cuticle, epidermis, and portions of the palisade parenchyma rolled dramatically within several minutes as it dried (Figure 7B—arrow). When water was added to the rolled adaxial portion of the leaf, the tissue flattened to its former shape within seconds (not shown). Though it cannot be fully ensured that all of the spongy parenchyma had been removed nor that how much of the palisade parenchyma layer(s) were exposed since there is some overlap between the various layers within leaves (Figure 7A), but most, if not all of the spongy parenchyma was excluded. Therefore, while desiccation-induced shrinking of the framework of the spongy parenchyma may be involved in leaf rolling, it likely does not completely account for the rolling of leaves when desiccated.

The adaxial surface of the leaf of *R. maximum* consists of a cuticle layer and a bilayer epidermis. Rolling of the manually isolated adaxial epidermis, possibly with some of the palisade (Figure 7B), suggests anisotropic contraction by epidermis versus palisade parenchyma or between the two epidermal layers themselves, due, presumably, to differential architecture, composition or extent of their cell walls (see more under "paper bilayer model"). It could also be the result of a cuticle that, by its nature, is resistant to dehydration but is relatively flexible and was induced to roll by the dehydration (and subsequent shrinkage) of attached epidermal and/or palisade cells below the cuticle. Adaxial cuticle deposition has been shown to increase significantly in cold acclimated leaves of rhododendrons, particularly more so in a thermonastic species relative to a nonthermonastic one (Wang et al., 2008).

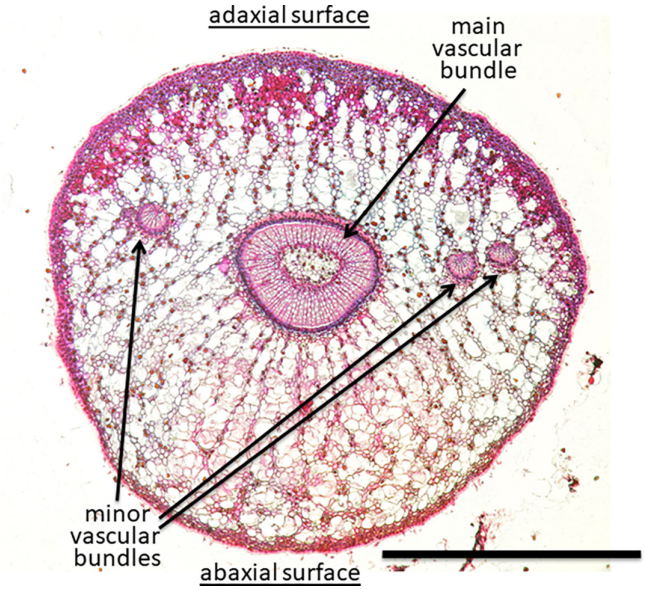

FIGURE 9 Cross section of an *Rhododendron maximum* petiole. Notice the thicker layer of cells along the adaxial surface. Bar is 1 mm.

3.3.2 | Petiole

Petiole curling intensified only after the vascular system froze and leaf rolling occurred (Figure 4G,H; Video S4). Notably, when a fresh petiole was cut longitudinally and allowed to dry, the adaxial half of the petiole curled noticeably downward while the abaxial half remained straight; when the dry, curled half of the petiole was placed in water, it became completely straight (not shown). This suggests petiole

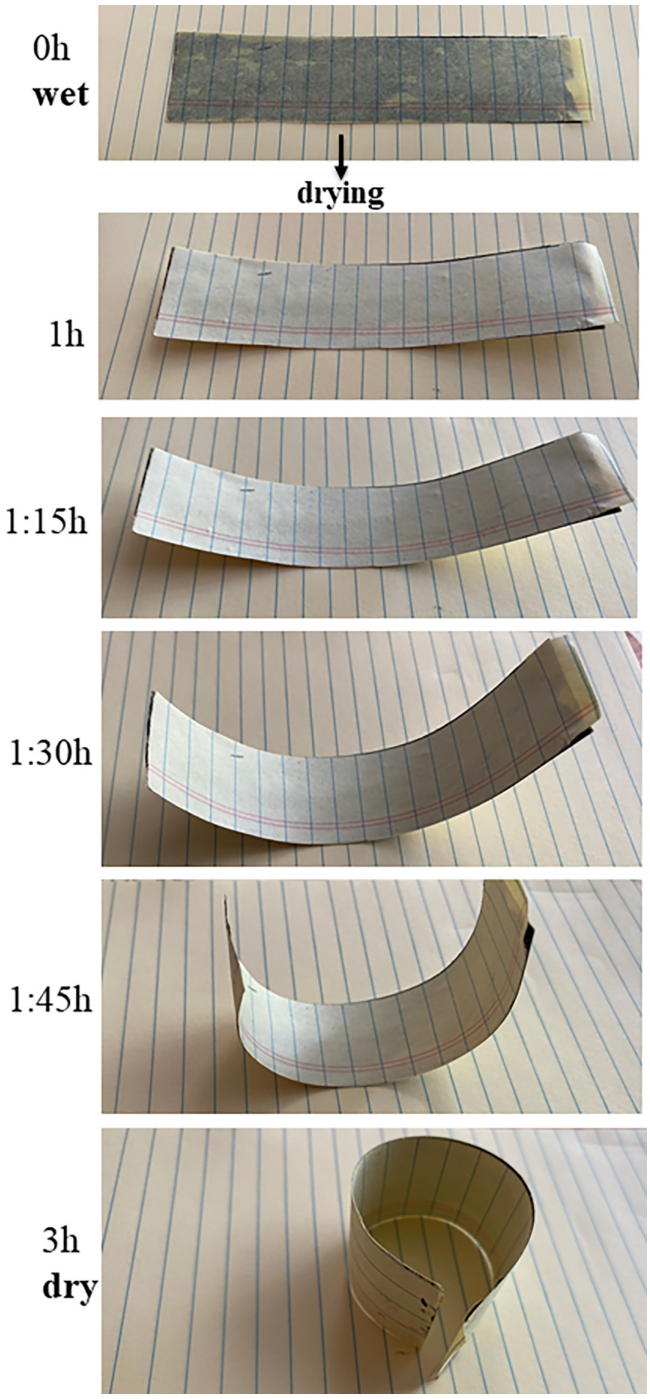

FIGURE 10 Paper bilayer made by cutting strips of paper in two directions and after wetting them joining the two layers with silicon caulk. The two layers differed by 2.4% in their coefficient of moisture expansion. As the layers dried, the shrinkage in the expanded layer caused the bilayer to roll like the *Rhododendron maximum* leaves did when they were allowed to dry. When the bilayer was placed back in water it became straight again (not shown).

curling after freezing was also due to dehydration. Anatomically, cells on the abaxial side of the petiole appeared similar to those on the opposite side. However, cells on the adaxial side are more compressed (substantially more so on the upper region of the adaxial semi-circle), whereas those on the abaxial surface are more sparsely spaced (Figure 9). Hence, even though the area of the abaxial side appears larger, the actual quantity of cells on the adaxial side is ostensibly much greater. Consequently, the movement of water vapor from cells on the adaxial surface to the vascular system (site of ice) must traverse across more layers overall, as well as through more cells distal to the vascular system than those proximal to it. This suggests anisotropy of freeze-desiccation induced contraction among the multiple layers of cells within the adaxial side as well as between adaxial and abaxial regions of the petiole (Figure 9). We propose that this anisotropy, coupled with more intercellular space for cell shrinkage in the abaxial region, causes stronger curling of petiole post-freezing.

3.3.3 | Paper bilayer model

To simulate the rolling effect of dehydrated leaves using a cellulose-based system, a paper from a lined tablet was cut in two directions to see if it possessed anisotropic characteristics. The paper that was cut with the lines expanded by 2.4% when wetted, and that cut in the perpendicular direction did not expand upon wetting. When the two damp slices of paper were joined using silicon adhesive and allowed to dry, within 3 h, the bilayer rolled (Figure 10) similar to leaves that were either frozen or allowed to air dry. Interestingly, a mere 2.4% contraction during drying that was opposed by the layer that did not change, caused the rolling effect shown in Figure 10. In rhododendron leaves this suggests that the numerous cells within whichever layer(s) are driving leaf-rolling would each need very little shrinkage from freeze-dehydration to cause the rolling observed following a freeze event.

Besides cellulose being a dominant structural component of leaves, intact cells within leaves bear little resemblance to a layer of isolated cellulose fibers. Remarkably, a simple bilayer of cellulose fibers arranged perpendicular to each other would behave nearly identically to a leaf consisting of intact cells. Owing to its hydroxyl functional groups, cellulose has a strong attraction to water and water intercalating between cellulose fibers causes sheets of cellulose to expand (Khazraji & Robert, 2013). Conversely, when water is removed the sheets contract. The interaction of water with cellulose is somewhat more complex, involving dispersion forces and volume (three-dimensional) changes (Khazraji & Robert, 2013), while for the "paper bilayer" in the present study, changes in only two dimensions are being considered.

Above discussion leads to a question: how much leaf-rolling is purely a mechanical result of dehydration and the anisotropic expansion/contraction of cellulose fibers as water moves in and out of microfibrils versus an active biological mechanism? Likely, it involves both mechanical and biological processes, the combination/contribution of which would vary depending on the environmental stress encountered. For example, under freezing conditions, active biological water transfer (via aquaporins) may predominate; Chen et al. (2013) have reported a substantial upregulation of *Rhododendron* leaf aquaporins (*PIP2*;1 and *PIP2*;2) coincident with leaf freezing/ice-

nucleation, however, under drought conditions a more mechanical process may overshadow a biological system. In fact, Wang et al. (2020) attempt to link a mechanical analysis of anatomical adaptations ("cellular scale architecture") and biological basis for thermo- and hygronasty that lead to anisotropic expansion of cell layers in leaves resulting in leaf rolling and petiole curling. Thickening and altered composition of plant cell walls during cold acclimation are well established (Stefanowska et al., 1999 and references therein). Moreover, a 47-fold upregulation of a gene encoding coumarate 3-hydroxylase, a key enzyme in the lignin biosynthesis pathway, has been reported to occur during cold acclimation in *Rhododendron carawbiense* (Wei et al., 2006). Cell wall rigidity favors resistance to cell collapse (cytorrhysis) and lowers cellular pressure potential, which in turn can reduce freeze-desiccation (Anderson et al., 1983). Therefore, conceivably, differential cell wall rigidity of the adaxial (palisade) versus abaxial (spongy parenchyma) sides could also contribute to the anisotropy of freeze-desiccation in *R. maximum* leaves. In support of this hypothesis, a recent study involving 11 herbaceous and woody species, including evergreens, noted decreased freeze-desiccation of mesophyll cells in those species with higher cell wall thickness and rigidity and smaller intercellular spaces (Stegner et al., 2022).

Recently, Takahashi et al. (2021) investigated the restructuring of cell walls during cold acclimation and made a case that cell wall remodeling is critical for the survival of tissues that were frozen. Membrane-bound kinases reportedly stimulate signaling cascades that promote wall compositional changes during cool temperatures, which in turn influence how cells respond to freezing stresses. Changes in the composition of the components of cellulose microfibrils reportedly have a significant effect on how cells deform during freezing (Takahashi et al., 2021). If these changes differ between the separate layers of the leaf, then, as Nilsen (1992) points out, differential shrinkage could result in the rolling behavior of leaves after they freeze.

3.3.4 | Freezing the bilayer

When the bilayer was frozen, it rolled just like it did when it dried (not shown). In fact, as in leaves, rolling did not occur until after the paper froze. In this case, the coil of the freezer had the lowest chemical potential of the system, and the paper rapidly dried after freezing, with rolling occurring simultaneously. (When the paper was coated with Vaseline to prevent desiccation, it froze at a colder temperature and did not roll). The gradient, in this case, was primarily a result of a difference in temperature between the coil and the bilayer. While in rhododendron leaves, the entire leaf is presumably at the same temperature, the gradient within leaves depended on a water potential difference between frozen and supercooled water.

3.4 | Differences between thermonasty and hygronasty

During freezing that is above the killing temperature, cells' membrane semi-permeability is intact, and they can regulate the movement of water in and out, presumably by the action of aquaporins, so leaf rolling and unrolling is likely a result of intact cells contracting and expanding, respectively (Arora et al., 2021). However, when the leaf is dead, it still rolls and unrolls, even though cells are incapable of active water transport. Along with larger intercellular spaces in the spongy parenchyma, this likely involves a differential CME of cellulose (walls of dead cells) with water by adaxial versus abaxial surface of leaves (similar to the paper bilayer model) (Miyake et al., 2000; Schroeder, 1972). Wang et al. (2020) uses the phrase "anisotropic distribution of the expansion coefficient," a conceptually valid explanation. Although, in the case of leaf rolling and petiole curling, the expression should probably be considered a "shrinkage coefficient."

Water movement in both hygronasty and thermonasty is likely facilitated by aquaporins (Chen et al., 2013). However, the difference between the two nastic effects is that hygronasty results from the loss of water primarily to the outside environment (stomatal/cuticular transpiration) and leaves recover by water being supplied through the root, stem, and leaf vascular system. Thermonasty, on the other hand, ostensibly involves water movement out of cells along a gradient to a region of lower water potential but within the leaf, namely to sites where ice is present, that is, the vascular bundle. When the leaf is thawed, the gradient is reversed, and water moves from the vascular bundle back into contracted cells, turgor is restored, and the leaf unrolls. Wang et al. (2020) reported that when petioles were removed from leaves, the extent of rolling was reduced, providing evidence that water transport from the petiole was involved in leaves unrolling when thawed. This could be a concurrent supply of water to unroll the leaf in addition to water from the reversal of the gradient.

In thermonasty, water is not lost from the leaf to the environment, at least to the extent that it is in hygronasty. Because the symptom (leaf rolling) of both effects is the same, it is tempting to draw parallels between the causes of the rolling that may not be supportable. It could be argued, however, that the ultimate cause is the same in both nastic effects, namely a difference in water deficit between the upper and lower regions of leaf surfaces.

4 | CONCLUSION

The nonthermonastic *Rhododendron ponticum* is reportedly less freezing tolerant than *R. maximum*. In fact, Nilsen (1991) reported a correlation between freezing tolerance and the extent of rolling in *Rhododendron* species, with hardier species exhibiting greater intensity of rolling. It may be possible to apply the study of hygronasty or thermonasty in *Rhododendron* to crop plants such as maize, rice, wheat, oats, and barley. Even though these crop plants are monocots, they behave similarly under drought and freezing stress, with hardier crops (rye) rolling significantly more when frozen than less hardy oats (our unpublished results). Identifying simple screening tools to develop more freezing or drought tolerance genotypes will benefit breeders in their attempts to breed more abiotic-stress tolerant cultivars. The tissues involved in *Rhododendron* provide an excellent model system because they are much larger and, therefore more easily observed and manipulated. In addition, determining reasons for a lack of curling in the less freezing tolerant and

nonthermonastic *R. ponticum* could provide a means to understand freezing tolerance in other species, such as small grains.

AUTHOR CONTRIBUTIONS

Rajeev Arora and David Livingston jointly conceived the idea and designed experiments. David Livingston and Tan Tuong primarily conducted the experiments. Rajeev Arora and David Livingston analyzed the data and led the manuscript writing in consultation with Michael Wisniewski. All authors contributed to editing.

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DATA AVAILABILITY STATEMENT

All the data and supplementary materials for supporting the findings of this investigation are available from the corresponding author upon the reasonable request.

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