

# Rhododendrons International

*The Online Journal of the World's Rhododendron Organizations*



Vireyas



Rhododendrons



Azaleas

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# Contents

ii From the Editor, *GLEN JAMIESON*

## PART 1

### **Introduction**

- 1** *CHAPTER 1. The Rhododendron Research Network: Promoting and Enhancing Rhododendron Research through Interdisciplinary Collaboration and Public Engagement* JULIANA S. MEDEIROS AND ERIK T NILSEN

### **Physiology**

- 8** *CHAPTER 2. A mechanistic Understanding of Winter Hardiness in Rhododendron: Implications in Breeding for Cold Hardiness* RAJEEV ARORA

### **Physiological Ecology**

- 27** *CHAPTER 3. Mini-review of Rhododendron Ecophysiology* ERIK T NILSEN
- 43** *CHAPTER 4. Altitudinal Trends of Efficiency and Stability of Water Transport in the Stems and Leaves of Nepalese Rhododendron Species, Based on Morphological Traits* HARUHIKO TANEDA AND SUICHI NOSHIRO

## PART 2

### **Evolutionary Ecology**

- 66** *CHAPTER 5. The Unique Value of Genus Rhododendron for Investigating the Evolutionary Ecology of Root-microbe Interactions* JULIANA S MEDEIROS, YU LIU AND JEAN H BURNS

### **Genetics and Evolution**

- 82** *CHAPTER 6. Floral Morphology of Rhododendron and its Relation with Pollinators* EAPSA BERRY AND R GEETA
- 98** *CHAPTER 7. A Look at Hybrid Zones in Rhododendron, with particular Reference to F1-Dominated Hybrid Zones (F1DZs)* RICHARD I MILNE

### **Horticulture**

- 118** *CHAPTER 8. Where Senses and Science Converge* STEVE KREBS

### **Ecology & Ethnobotany**

- 126** *CHAPTER 9. Monitoring Rhododendron Flowering Times in a Changing Climate* ROBBIE HART AND SAILESH RANJITKAR

### **Medicinal Chemistry**

- 141** *CHAPTER 10. Rhododendron Natural Products as Sources of Novel Antibiotics* NIKOLAI KUHNERT, INAMULLAH HAKEEM SAID, ABHINANDAN SHRESTHA, AHMED REZK, ANNE GRIMBS, JENNIFER NOLZEN, HARTWIG SCHEPKER, KLAUDIA BRIX, DIRK ALBACH AND MATTHIAS ULLRICH

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# From the Editor

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*Rhododendrons International (RI)* is an online journal distributed free to all the world's known rhododendron associations for their internal distribution. It can also be accessed without charge on the American Rhododendron Society website at <https://www.rhododendron.org/ri-index.htm>. This third volume of *RI*, broken into two parts because of the large size of the articles, contains the first series of research articles from the Rhododendron Research Network (R-RN), which is a collaboration between the American Rhododendron Society (ARS) and an international group of rhododendron researchers. More detail about the objectives of this network and what is contained in this volume are given in the introductory chapter by the network's co-chairs.

This network is an exciting collaboration between the ARS and the world's rhododendron researchers, and we hope that other rhododendron societies will also actively engage in this initiative by widely sharing this volume with botanists in their own countries and encouraging them to also become participants. I look forward in the future to receiving more dedicated research articles and producing another *RI* volume to further this endeavour.

*Section Introduction*  
*Chapter 1*

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# **The Rhododendron Research Network: Promoting and Enhancing *Rhododendron* Research through Interdisciplinary Collaboration and Public Engagement**

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J. Medeiros



E. Nilsen

On behalf of the members of the Rhododendron Research Network, we welcome you to this research edition of *Rhododendrons International*. The Rhododendron Research Network (R-RN) is a collaboration between the American Rhododendron Society (ARS) and an international group of other rhododendron researchers seeking to: 1) promote *Rhododendron* as a study system in biology, 2) promote the ARS, associated botanical gardens and rhododendron societies as a resource for *Rhododendron* research and public engagement, 3) foster participation and membership of scientists in the American Rhododendron Society, and 4) create opportunities for communication and collaboration within the international community of researchers who study *Rhododendron*, and between those scientists and non-scientist ARS members.

As our first steps in pursuit of these goals, we established a website (Rhodo-Research.net) as a central point of communication for all network activities, and we solicited articles from researchers working on *Rhododendron* to be published here in *Rhododendrons International*. We hope that this issue will benefit researchers by expanding their breadth of knowledge, and by sparking inspiration for interdisciplinary studies. In addition, we aspire to make cutting-edge research accessible for use and enjoyment by *Rhododendron* enthusiasts and public garden practitioners.

### **What is a model study system, and why is it important for innovation?**

The model study system concept rose in popularity over the 20<sup>th</sup> century as the volume and complexity of scientific knowledge began to dramatically increase. There are over 200,000 plant species on earth, and for all of them, many factors intermingle to determine their growth and survival, and how they interact with each other. Model systems identify a focus group with broad appeal, one which can provide general insight about important biological processes, and in which many scientists are working together to make advances.

*Arabidopsis thaliana* [Mouseear cress, a member of the mustard (*Brassicaceae*) family, which includes cultivated species such as cabbage and radish], the most famous plant study system, is an annual herbaceous plant. It is easy to grow, small in stature, has a short life cycle, and it can be genetically manipulated. Within this system, important advances have been made in understanding the genetics of plant growth regulation, photosynthesis and phenology (the timing of life events like blooming). As vital as *Arabidopsis* has been in advancing research in plant physiology and genetics, the narrow ecological range of the genus, specialized annual life cycle, and relatively low phenotypic diversity among species in the genus, limit the ability to draw insight into ecological and evolutionary processes that drive plant biodiversity. In addition, *Arabidopsis* itself has no economic significance outside of the role it plays in research. This is important, in part, because it slows the direct application of findings in *Arabidopsis*, but also because this obscurity restricts its usefulness in engaging public audiences as consumers of scientific information.

The importance of scientific public engagement for modern research programs cannot be understated. As the technological complexity of our society increases, the public needs to be informed about scientific advances, and the first thing people want to know is: how does this directly affect my life? In support of this, funding agencies that are sustained by public funds (e.g., United States of America National Science Foundation) are increasing efforts to vet proposals from this perspective, such that the ability to offer public engagement is fast becoming a central factor in the awarding of research funding. Thus, public interest should be considered as an important facet of

study system development, but this aspect has rarely been satisfied in current biological study systems.

For these reasons, new study systems are needed which address tenacious questions that cannot be robustly applied to existing systems like *Arabidopsis*, and which have the charisma to engage public audiences more fully as stakeholders in science.

### **Why promote *Rhododendron* as a study system?**

*Rhododendron* is one of the most speciose and diverse genera of plants on earth, with over a 1000 species, inhabiting environments from the Arctic to the tropics, ranging in size from small alpine shrubs to tropical epiphytes, to large trees, and being both deciduous and evergreen. As a result, this genus represents a rich study system for addressing a wide range of questions in functional biology. *Rhododendron* has also played an important role in human culture. The book *A Brocade Pillow* by Ito Ihei (1692; republished as Ito and Creech (1984)) initiated the long history of horticultural research interest in this genus, going back over 325 years, and substantiates ongoing research in both realized and potential human use of *Rhododendron*. Therefore, *Rhododendron* both meets the need for a new study system that can address difficult questions in organismal biology and provides the required charisma and citizen science mass to engage the public in science. As described in Volume 1 of *Rhododendrons International*, there are 16 world rhododendron organizations, collectively with upwards of 5000 members, spread from northern Europe to North America, to New Zealand and Australia, and to India, China and Japan.

Below, we summarize the areas of research covered in this edition of *Rhododendrons International*, which represent some of the topics where research on *Rhododendron* has already provided unique insight. Importantly, the works presented here speak to the interplay of basic and applied approaches, and to the importance of interdisciplinary studies. We have the potential to push the boundaries of plant biology and drive scientific innovations by combining approaches in physiology, ecology, evolutionary biology, ethnobotany and genetics with this woody plant. Moreover, we can address modern problems faced by humankind if our research includes aesthetic, medicinal and cultural relevance. Part 1 has articles on Physiology and Physiological Ecology. Part 2 has articles on Evolutionary Ecology, Genetics and Evolution, Horticulture, Ecology and Ethnobotany, and Medicinal Chemistry.

### **Research in Functional Biology:**

As briefly mentioned above, the 1000+ species in genus *Rhododendron* are broadly distributed across both latitude and elevation in the montane regions of the northern hemisphere, from the Arctic to the tropics. Arctic and alpine species tend to be short and

scrubby survivors, while species of temperate habitats can reach the size of large trees, and epiphytes are an important growth form in tropical habitats where competition and biodiversity are extensive. Within these climates, rhododendron species play a variety of ecological roles. Some species dominate their native floras, others are members of declining endangered populations, and still others are known to be aggressively invasive when introduced into new areas.

Two important aspects of *Rhododendron* diversity are the wide range of physiological cold hardiness across species, and their high diversity in leaf form. This has attracted researchers interested in temperature stress physiology. In this issue, Arora (Chapter 2) summarizes a substantial body of research focused on the physiological mechanisms by which evergreen leaves withstand freezing, as well as the genetic mechanisms of frost survival. Another important avenue of physiological investigation in *Rhododendron* has been defining how temperature interacts with drought to impact water transport, and how this in turn determines leaf function. In Chapter 3, Nilsen shows that inherently low water transport capacity in the wood of all *Rhododendron* sets up unique physiological challenges for their leaves. This chapter also highlights a fascinating array of discoveries concerning the water-saving solutions found across different species and climates.

By examining *Rhododendron* growing at different altitudes in Nepal, Taneda and Noshiro (Chapter 4) demonstrate that species must navigate a physiological trade-off between growing fast and being stress resistant, and they do so by altering many traits simultaneously, such as by adjusting growth form, leaf size, and the water transport properties of wood and leaves. Variation in the relationships between plant physiology and ecological conditions has far-reaching consequences, as small-scale patterns of growth and survival directly influence large-scale population dynamics, providing the raw material for evolution by natural selection.

In addition to elucidating variation in physiological responses, determining which traits represent functional adaptations also requires understanding the underlying causes of that variation, and determining whether or not traits have relevance for fitness. In pursuit of this, many studies have focused on the substantial leaf diversity in *Rhododendron*. In this regard, species of *Rhododendron* have often been thought to be quite similar morphologically and physiologically below ground, based on their shared propensity to thrive in acidic, low-productivity soils. In Chapter 5, however, Medeiros *et al.* provide compelling evidence for extensive morphological diversity in *Rhododendron* roots, which coincides with an extraordinary diversity in the types of their associated soil microbes. This suggests that roots and soil microbes are important components in the evolution of diversity within the genus. Another important relationship, that between plants and their pollinators, is emphasized by Berry and Geeta (Chapter

6), who focus their observations on the fascinating diversity of *Rhododendron* floral morphology, showing both prevalent and rare floral forms, and that particular forms are associated with particular pollinators, e.g., butterflies, birds or bees. Still, one of the most confounding factors in understanding *Rhododendron* evolution is likely to be their strong propensity for hybridization. Milne (Chapter 7) describes the puzzling cases where wild rhododendrons occupy distinct zones populated only by first-generation hybrids, outlines the improbability and potential causes of this phenomenon, and discusses how study of these F<sub>1</sub> hybrid zones can provide unique insight into ecological and evolutionary processes.

### **Research in Plant-Human Interactions:**

The importance of *Rhododendron* to humans is affirmed by the fact that botanical gardens and plant societies like the ARS boast a long history of supporting research on the genus, and consequently these organizations harbor a wealth of knowledge and resources. Many young scientists today are seeking to engage the public on some level, but have little familiarity with public organizations like the ARS, probably due to historically insular attitudes and training practices within the scientific culture itself. Chapters 2-7, described above, highlight efforts to understand fundamental problems in *Rhododendron* biology and ecology that are relevant for breeders, shining a light on the important interplay between basic and applied research. In Chapter 8, Krebs outlines extensive ARS activities in support of research, and the strong benefits to scientists in incorporating citizen science into research programs through working with organizations like ARS. As another example of how important this plant genus is to humans, native *Rhododendron* features are known to influence cultural patterns and decisions. For example, in Chapter 9, Hart and Ranjitkar provide a fascinating account from the Hengduan Mountains of China, where the sequential flowering of native *Rhododendron* species has served for many generations as a colorful and reliable calendar to track the timing of important events in daily life, such as the planting of buckwheat. Their work also demonstrates that climate warming is altering these patterns, and suggests that the seasonal timing of flowering (phenology) for some species could become mismatched with the timing of relevant climate conditions for growth and/or pollination. These changes will clearly have consequences for *Rhododendron* population dynamics, but they may also impact humans, particularly if important species become endangered or extirpated as a result.

The risk of losing species, and the full value of *Rhododendron* as an interdisciplinary study system, becomes particularly prescient when considering their potential in medicine. Perhaps as an extension of their physiological and ecological diversity, *Rhododendron* produce a wide variety of secondary chemicals, which are critical in

plant responses to stresses such as drought, herbivory, or pathogen attacks. Though these chemicals are often toxic to humans, Hart and Rajitkar (Chapter 9) highlight diverse ethnobotanical uses of *Rhododendron*, and Khunert *et al.* (Chapter 10) show the great potential of secondary chemicals from *Rhododendron* species as a source of new antibiotics. Khunert *et al.* also note that the possible applications of chemicals derived from *Rhododendron* are only beginning to be explored, bringing us back to the need for continued and concerted efforts to better understand *Rhododendron* ecology and by default, chemistry.

### **Looking to the future with the Rhododendron Research Network**

The fascinating research described here bridges genetics, ecology, and evolutionary biology, and offers great promise in solving many complex problems in functional biology. At the same time, *Rhododendron* consumers and enthusiasts stand to benefit greatly from the application of these findings to problems they care about, such as the development of new and improved plants for home landscapes, conserving *Rhododendron* species in the wild, and the discovering of new medicines.

But what frontiers await? Investigations into all of these research areas will be greatly enhanced in coming years by improved genetic tools. For example, separate efforts to fully sequence reference genomes of *Rhododendron* are now coming to fruition in the United States and China. Few woody plant genomes are available for study, so *Rhododendron* is now poised to become a preeminent study system in genetics as well. Because of their high diversity in physiological traits and climate, comparative studies among species in *Rhododendron* have the potential to provide pivotal insight into the genetics of stress tolerance/resistance, an area of particular relevance for climate change, but not particularly amenable for study in *Arabidopsis*. Beyond this, genetic manipulations (e.g., gene knockouts and gene over-expressers) in *Rhododendron* would improve our understanding of how putative stress tolerance/resistance genes influence physiological functions.

The underlying ecological and taxonomic diversity of *Rhododendron*, when combined with an understanding of their genetics, will open up many applications. In addition, the long history of human fascination with *Rhododendron* is evidence of their overarching importance to human culture and society, a value that cannot be understated in the current scientific climate. But how do we make the most of this exciting and dynamic study system? Our Rhododendron Research Network is designed to jump start advances in *Rhododendron* by facilitating interactions, breaking down barriers, and bringing both scientists and gardeners with diverse backgrounds together

in the spirit of interdisciplinary science. We hope that this *Rhododendrons International* volume will inspire a wide variety of people, researchers and lay persons alike, to get involved in this initiative. We invite you to visit our Rhododendron Research Network webpage at [www.rhodo-research.net](http://www.rhodo-research.net) to learn more about our network initiatives, and to find out how you can hopefully participate.

## **Reference**

Ito, I., and J.L. Creech. 1984. *A Brocade Pillow: Azaleas of Old Japan*. Weatherhill, New York: 161 pp.

*Juliana S. Medeiros and Erik T. Nilsen, as well as being Co-Chairs of the Rhododendron Research Network, are scientists at the Holden Arboretum, Kirtland, OH, and Virginia Tech, Blacksburg, VA, respectively, and are members of the Great Lakes and Middle Atlantic ARS chapters, respectively.*

# **A Mechanistic Understanding of Winter Hardiness in *Rhododendron*: Implications in Breeding for Cold Hardiness**

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## **Introduction**

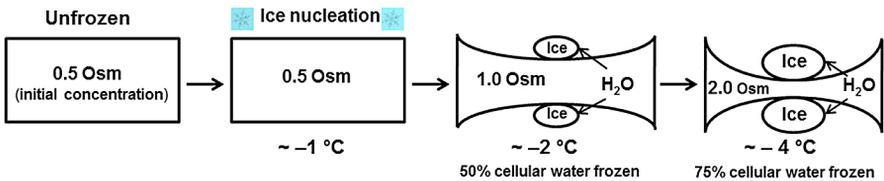
Freezing damages plants by causing both dehydration and mechanical injury to cells. Winter-survival of landscape perennials in temperate and boreal zones involves changes in the various components of winter-hardiness, including: 1) cold acclimation, 2) deacclimation and rehardening, and especially for evergreens, 3) photo-protection. Improvement in the tolerance to freezing of woody plants such as rhododendrons to increase their suitability for culture in colder climates, largely involves classical breeding for specific characteristics. A mechanistic understanding of the components affecting freezing tolerance could improve these breeding efforts.

Additionally, these physiological processes are highly coordinated with climatic cues, and thus may interact with climate change to produce unexpected effects of freezing damage. Our laboratory has conducted physiological and biochemical studies on these components of winter-hardiness with *Rhododendron* species and cultivars. Here, we present a synopsis of some of this research, and conclude with thoughts about some unresolved questions. But first it may be useful, especially for the uninitiated, to briefly review the process of freezing and the resultant stresses experienced by plant cells when exposed to below freezing temperatures.

## **Freezing and its associated stresses and injury in plants at the cellular level**

During a frost episode, ice typically forms first in the apoplast (i.e., the space lying outside the plasma membrane consisting of intercellular spaces along with the cell wall) of plant tissues, which has a higher temperature freezing point than the cell sap. Upon freezing at any given temperature, the vapor pressure of ice is less than that of a liquid

solution, and this difference increases as temperature decreases (Hansen and Beck 2008). Thus, once extracellular ice forms, water molecules move to regions of lower vapor pressure, i.e., from the protoplasm through the plasma membrane via osmosis, leading to cellular dehydration (Fig. 1). The net amount of dehydration depends on both the initial solute concentration of the cell sap and the subfreezing temperature, because these determine the vapor pressure differential across the plasma membrane. For example, assuming an initial cell sap concentration of approximately 0.5 osmolar, freezing at  $-4^{\circ}\text{C}$  will remove about 75% of the osmotically active water from the cell. In contrast, the same freezing removes about 50% of the freezable water if the initial cell sap concentration is one osmolar (Fig. 1).



**Fig. 1.** Diagrammatic representation of cell collapse due to cellular dehydration during extracellular freezing.

Physical stress (mechanical shearing) from the presence of ice in the apoplast can also damage plant tissues. Cell membranes can thus be disrupted during freezing both by desiccation and mechanical stresses. Consequently after thawing, the cellular contents can leak out from plant tissues. The amount of leakage is a function of the magnitude of stress, which is the result of both the extent of freeze-desiccation and the duration of freezing. Quantitatively this is measured using an “ion- or electrolyte-leakage test” (Dexter *et al.* 1932), which remains the most widely used method to estimate the freezing tolerance of tissues. Conventionally, the freezing tolerance of a plant tissue is defined as the Lethal Temperature that results in 50% ion-leakage/injury, i.e., LT50, which is also strongly correlated with the temperature at which the rate of injury is maximum (Tmax; Lim *et al.* 1998a). A cold acclimated plant has a much lower (more negative) LT50 than its non-acclimated counterpart (Fig. 2A), as the threshold tolerance of tissues to freeze-desiccation shifts to colder freezing temperatures during the cold acclimation process (Fig. 2B). The physiological and/or biochemical adjustments by cells that enable such a shift has been a topic of investigation in many laboratories, including ours.

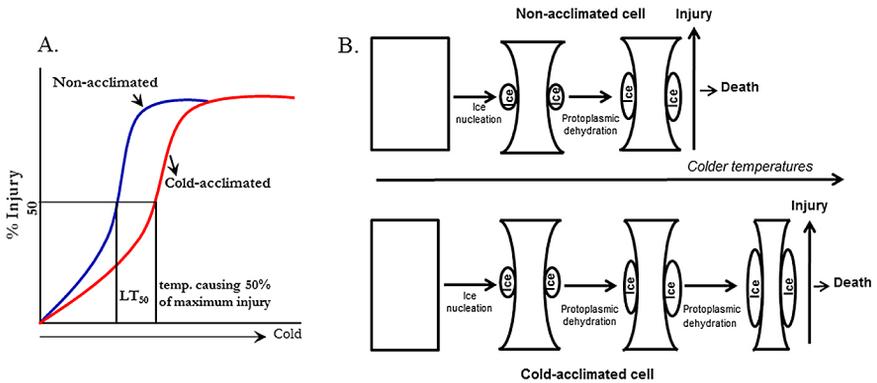


Fig. 2. (A) A stylized diagram representing a relatively lower and higher freezing tolerance, expressed as  $LT_{50}$ , of non-acclimated and cold-acclimated plant tissues, respectively; (B) Diagrammatic representation of the shifting of the “tolerance threshold” for freeze-induced cellular dehydration to relatively colder temperatures after cold acclimation.

### Components of winter-hardiness

Winter-survival of landscape perennials in temperate and boreal zones involves a multi-component strategy during their annual cycle (Fig. 3).

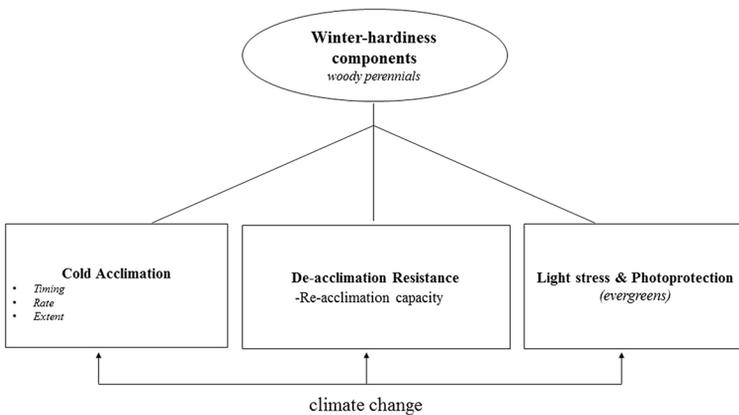


Fig. 3. Some of the key components of winter-hardiness in evergreen perennials that can be impacted by the vagaries of climate change.

**Cold-acclimation:** Cold-acclimation, the first line of defense in a protection strategy, involves the incremental acquisition of freezing tolerance by non-acclimated

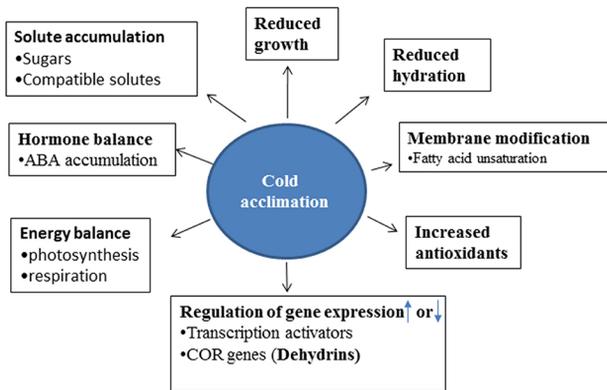
plant tissues over weeks and months through the autumn, to become maximally hardy by winter (Weiser 1970). Besides the absolute gain in freezing tolerance, when (the timing) and how efficiently (the rate) cold-acclimation occurs are also critical (Fig. 3). In most woody plants, the first stage of cold-acclimation is induced by a shortening day-length after growth cessation. This partial cold-acclimation is followed by stage two, a more substantial increase in freezing tolerance in response to cold exposure (above and below freezing) during the autumn and winter (Weiser 1970, Arora *et al.* 1992). A mechanistic understanding of the cold-acclimation process, which is a genetically-determined, complex response mediated by a myriad of physiological and biochemical changes in plant tissues (Xin and Browse 2000), could provide us with biochemical/physiological markers for a freezing tolerance or cold-acclimation ability that may be useful as screening tools in plant breeding efforts. It should also be recognized that cold-acclimation ability, i.e., the degree of freezing tolerance gained, could be influenced by the plant's age, both physiological and chronological (McNamara and Pellet 2000; Lim *et al.* 2014), and therefore one may not necessarily be able to predict the freezing tolerance potential of a mature plant in the landscape based solely on the phenotypic selection of young seedlings.

**Deacclimation resistance:** The freezing tolerance that is acquired during cold-acclimation is lost in response to warming or spring-like conditions during the annual cycle via a process called deacclimation. It may seem counterintuitive, but deacclimation kinetics, i.e., when, how much, or how fast de-acclimation occurs, can significantly impact overall winter-survival of woody plants, including rhododendrons (Kalberer *et al.* 2006). Deacclimation response is of particular significance in the context of climate change scenarios. Erratic temperature fluctuations, for example, the occurrence of spring-like conditions followed by a hard freeze, could damage prematurely deacclimated tissues and cause winter kill. Indeed, the frequency and magnitude of such fluctuations are on the rise (Jentsch *et al.* 2007; IPCC 2014) and some of the most devastating killer frosts across North America in recent years are attributed to such climate events, e.g., in the eastern USA, the Easter freeze of 2007 (Gu *et al.* 2007), the Mother's Day freeze of 2010, the killer frost of 2012, and the polar vortex of 2014. Field simulations of winter-warming events have also confirmed their damaging effects on overwintering perennials (Bokhorst *et al.* 2009, 2010). Despite a premature, partial deacclimation, plants can survive subsequent freezes if they are able to quickly and sufficiently reacclimate when cold conditions return. Reacclimation capacity, therefore, could be another important factor for winter survival by woody plants. Having an understanding of plant or environmental factors that influence deacclimation resistance and reacclimation capacity (Fig. 3) would be useful in our efforts to select and breed winter hardy genotypes, especially for their resiliency to sudden temperature extremes.

**Photoprotection:** Another component of winter-hardiness specifically relevant to broad-leaved evergreens is an ability to manage light exposure during the winter (Fig. 3). At freezing temperatures, photosynthetic biochemistry (enzymatic carboxylation and photosynthetic electron transport) becomes substantially slower, or even completely inhibited. However, green leaves continue to intercept solar radiation in winter, and in deciduous forests, winter light penetration to the understory is likely higher than that experienced during the growing season. Understory evergreens in the deciduous forests, such as rhododendrons, could thus potentially suffer from an energy input in excess of what can be processed by photosynthesis, rendering them vulnerable to photo-oxidative stress. Warmer winters, perhaps as a result of climate change, can exacerbate this situation by reducing the snow cover around short-statured evergreen shrubs, which otherwise would be insulated against excess light (Neuner *et al.* 1999). Below I also describe some of the various mechanisms that plants have evolved, anatomical and biochemical, to either tolerate or avoid light stress during winters (Adams *et al.* 2004, Wang *et al.* 2008). Insight into the photoprotection mechanisms will also be addressed as a somewhat less studied aspect of winter-hardiness.

**Cold acclimation physiology research**

As indicated above, cold acclimation is a multi-genic, integrated complex response involving changes in both the physiology and biochemistry of plant tissues. Several of these major changes are illustrated in Fig. 4. Some have to do with the adjustment of cellular metabolism to the biophysical constraints imposed by low temperatures, while others with the actual induction of freezing tolerance.



**Fig. 4.** Some of the commonly observed changes in cellular processes during cold acclimation.

Freezing has a strong effect on cellular water relations, and desiccation tolerance in plant cells is a key component of the cold-acclimation process. This involves the accumulation of compounds or molecules in cells that can mitigate desiccation or maintain hydration around membranes and other proteins (enzymes). We have tested this adaptation in a series of studies with *Rhododendron* species, cultivars, and breeding populations that vary in their freezing tolerance and cold-acclimation ability. In particular, we have focused on a class of highly hydrophilic proteins called dehydrins, which are thought to serve as “molecular sponges” that help retain hydration around cell membranes and proteins during a freezing stress, thereby protecting them from freeze desiccation injury (Close 1997, Kosová *et al.* 2007).

**The association of dehydrin expression with level of freezing tolerance in F<sub>2</sub> progeny:** We created an F<sub>2</sub> progeny array derived by first crossing a super cold-hardy parent (*R. catawbiense* ‘Catalgla’, ~40-year-old; leaf LT50 ~ -52° C) with a moderately-hardy parent (*R. fortunei*, ~40-year-old, LT50 ~ -31° C) that resulted in *R. catawbiense* × *R. fortunei* hybrids (LT50 ~ -43° C). We then self-pollinated these F<sub>1</sub> progeny to obtain an F<sub>2</sub> population. Leaf freezing tolerance of cold acclimated leaves from 51 F<sub>2</sub> progeny (3-year-old seedlings) and the parental plants were estimated by the ion-leakage method (see Lim *et al.* 1998b for detailed methodologies).

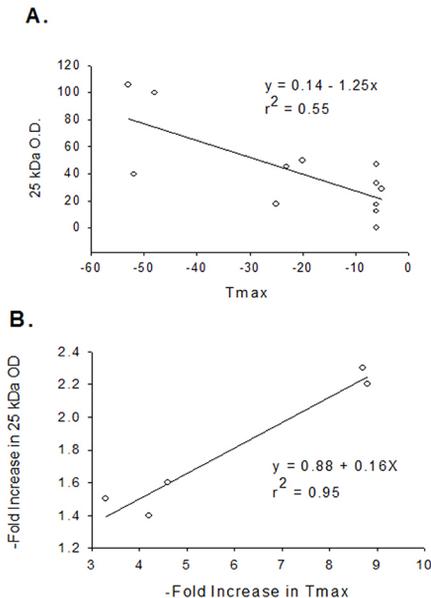
We found that a 25-kD dehydrin was the only protein clearly associated with differences in leaf freezing tolerance among F<sub>2</sub> seedlings, with a significant 50 to 100% increase in the 25-kD dehydrin level as the leaf freezing tolerance increased. The total amount of 25-kD dehydrin detected accounted for 78% of the variation in leaf freezing tolerance among parents, F<sub>1</sub>, and nine F<sub>2</sub> progeny (Lim *et al.* 1999), suggesting that the relative abundance of this dehydrin could serve as a genetic marker to distinguish between super cold-hardy and less cold-hardy *Rhododendron* genotypes.

Our study also provides some insight into genetic mechanism of inheritance: presence vs. absence (+/-) of the 25-kD dehydrin could be due to structural gene differences along with a dominant or co-dominant inheritance of dehydrin presence. Also, accumulation of several dehydrins in the F<sub>1</sub> were intermediate to parental levels, suggesting a gene dosage effect, which could result from co-dominant expression of “present” and “absent” alleles at the corresponding loci. We also detected higher molecular weight dehydrins uniquely contributed by the *R. fortunei* parent and present in the F<sub>1</sub> hybrid ‘Ceylon’, but these were barely detectable among F<sub>2</sub> progeny (Lim *et al.* 1999). The physiologically mature F<sub>1</sub> plants displayed only weak presence of these dehydrins in a cold-acclimated state.

**Dehydrin concentration variability among rhododendrons differing in their leaf freezing tolerances and geographic distributions:** In a study examining

21 species and cultivars, it was established that 11 dehydrins, including the 25-kD dehydrin, showed increased accumulation in cold acclimated leaf tissues, compared to non-cold acclimated plants (Marian *et al.* 2004). However, the 25-kD dehydrin was uniquely conserved across most (20 of the 21, or 95%) of the species surveyed (Table 1). The only *Rhododendron* species among those studied that lacked 25-kD dehydrin, *R. brookeanum*, a tropical Indonesian vireya with a low freezing tolerance ( $-7^{\circ}\text{C}$ ), which would not normally be exposed to very cold conditions in its native habitat. Non-cold acclimated leaves were not evaluated for freezing tolerance in this species, but previous work with various *Rhododendron* cultivars and species indicated that a non-acclimated leaf freezing tolerance within *Rhododendron* generally varied from  $-3^{\circ}\text{C}$  to  $-7^{\circ}\text{C}$  (Holt and Pellet 1981, Anisko and Lindstrom 1995, Lim *et al.* 1998a,b). Other tropical vireyas in section *Schistanthe* are typically injured by even slight freezing after cold acclimation (Sakai *et al.* 1986). In addition, the single dehydrin observed in *R. brookeanum*, a 41-kDa protein, did not appear to be increased in abundance in leaf tissues following fall acclimation (Table 1, see next page).

Regression analysis indicated a significant relationship between dehydrin abundance and leaf freezing tolerance across species, although a stronger relationship was obtained by standardizing the cold acclimated values relative to non-acclimated ones (Fig. 5). Results from this study reinforce those from our study with  $F_2$  progenies, further supporting our hypothesis that the 25-kD dehydrin plays a central role in *Rhododendron* cold hardiness, and suggesting that the accumulation of this particular dehydrin can be used to “predict” the extent of cold acclimation and subsequent leaf freezing tolerance.



**Fig. 5.** Association of freezing tolerance and 25-kD dehydrin abundance. (A) Regression of 25-kD O.D. on Tmax using absolute values (B) Regression of O.D. on Tmax using x-fold increase from nonacclimated to cold acclimated conditions ( $\Delta\text{OD}$  and  $\Delta\text{Tmax}$ , respectively) in leaves. O.D. = optical density, a quantitative measure of 25-kD band intensity; Tmax = temperature causing maximum rate of injury, a measure of leaf freezing tolerance (adapted with permission from Marian *et al.* 2004)

**Table 1.** *Rhododendron* species studied, their corresponding cold-acclimated leaf freezing tolerances, and various dehydrin proteins that accumulated in cold acclimated leaves.

Species	Leaf Freezing Tolerance C°	Dehydrins (kDa)										
		25	28	30	32	34	37	41	46	50	64	73
<b>Subgenus <i>Hymenanthes</i></b>												
<i>R. adenogynum</i>	(-29)	X								X		
<i>R. arboreum</i>	(-20)	X								X		
<i>R. brachycarpum</i>	-60	X	X						X	X	X	
<i>R. brachycarpum</i> subsp. <i>brachycarpum</i> (previously subsp. <i>tigerstedtii</i> )	ND	X	X						X	X	X	
<i>R. brachycarpum</i> 'Roslyn form'*	ND	X	X						X	X	X	
<i>R. catawbiense</i> 'Catalgla'	(-53)	X										
<i>R. decorum</i>	ND	X	X									
<i>R. dichroanthum</i> aff. subsp. <i>Scyphocalyx</i>	(-23)	X	X	X	X							
<i>R. fargesii</i>	ND	X								X	X	
<i>R. fortunei</i> 'Gable's hardy form'*	(-38)	X	X					X	X	X	X	
<i>R. fortunei</i> 'No. 27 DexterDexter'*	ND	X	X					X	X	X	X	
<i>R. makinoi</i>	ND	X		X				X		X		
<i>R. maximum</i>	(-52)	X		X						X		X
<i>R. maximum</i> (var. <i>rubrum</i> )	ND	X		X						X		X
<i>R. maximum</i> 'Mt. Mitchell'	ND	X		X						X		X
<i>R. metternichii</i>	(-48)	X	X							X		
<i>R. vernicosum</i> 'Gable's vernicosum'*	(-25)	X	X				X			X		
<i>R. degronianum</i> subsp. <i>Yakushmanum</i>	-40	X								X		

Table 1 continued.

<i>R. degronianum</i> subsp. <i>yakushmanum</i> 'Koichiro Wada'	ND	X								X		
<i>R. degronianum</i> subsp. <i>yakushmanum</i> 'Mist Maiden'	ND	X								X		
<b>Subgenus <i>Rhododendron</i></b>												
<i>R. brookeanum</i>	(-7)								X <sup>2</sup>			
<i>R. dauricum</i>	-50	X		X						X		
<i>R. hirsutum</i>	ND	X						X				
<i>R. keiskei</i> 'Mt. Kuromi'	-25	X						X				
<i>R. minus</i>	ND	X		X						X	X	
<i>R. mucronulatum</i>	-50	X				X				X		
<i>R. myrtifolium</i>	ND	X										
<i>R. russatum</i>	-40	X			X					X		

1. Leaf freezing tolerance values in parentheses were estimated in our laboratory as T<sub>max</sub> (the temperature causing maximum rate of injury), whereas those without parentheses are leaf lowest survival temperatures values from Sakai *et al.* 1986. ND = no data available. 2. No increase in cold acclimated leaves compared to the nonacclimated ones (the only case in this study where a dehydrin accumulation is not higher in winter collected leaves compared to summer collected ones. (Adapted with permission from Marian *et al.* 2004)).

**Ways dehydrins may confer freezing tolerance:** Studies examining the cellular actions of dehydrins typically take two approaches: 1) *in vitro* assays using a candidate dehydrin protein and another target protein (a cold-labile enzyme) to test if dehydrins could protect the target protein against deactivation by freezing or desiccation stress; and 2) transgenic overexpression of a candidate dehydrin gene in a model plant paired with a test plant to measure the gain in freezing tolerance over the non-transformed (wild-type) counterpart. We have used both of these avenues to test the functional relevance of a *R. catawbiense* dehydrin gene, RcDhn5. This gene was identified in our laboratory using expressed sequence tag analysis, which involved the comparison of cDNA libraries from cold acclimated (January) and non-acclimated (August) leaf tissues of field grown *R. catawbiense* 'Catalpa' plants (Wei *et al.* 2005). We demonstrated that RcDhn5 has cryoprotection and dehydration stress protection properties using *in vitro* assays (Arora *et al.* 2008, Peng *et al.* 2008a, Reyes *et al.* 2008). Briefly, the *in vitro* cryoprotection assay involved subjecting a cold labile enzyme to a freeze-thaw treatment either in the presence or absence of purified RcDhn5 (Arora *et al.* 2008). In the absence of RcDhn5,

the cold labile enzyme lost ~38% activity after one, and ~ 63% activity after three freeze-thaw cycles. In contrast, ~30 – 73% higher residual enzyme activity remained in the presence of RcDhn5 (Arora *et al.* 2008). Notably, parallel assays conducted with another hydrophilic polypeptide (but not a dehydrin) used in place of RcDhn5 did not protect enzyme activity (Arora *et al.* 2008), indicating that RcDhn5 can specifically protect enzymes from freeze-thaw deactivation. Similarly, when plants were exposed to controlled desiccation stress in the presence or absence of RcDhn5, RcDhn5 rescued enzyme activity, but the magnitude of this effect differed according to the level of water stress imposed (Peng *et al.* 2008a).

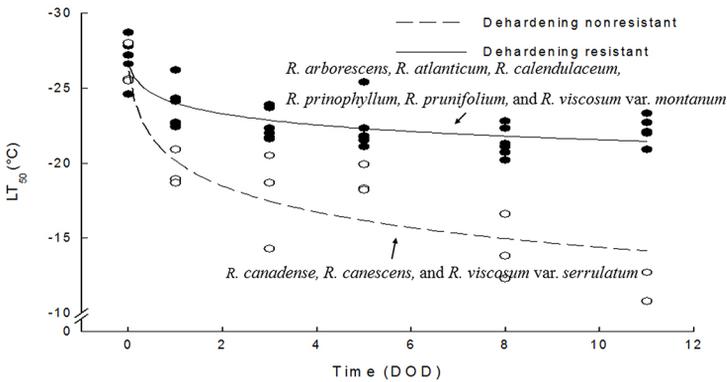
Transgenic studies in our laboratory involved overexpression of RcDhn5 in *Arabidopsis*, a model herbaceous plant, since a *Rhododendron* transgenic system is currently not available; detailed methodologies and results are presented in Peng *et al.* (2008a). Briefly, we found RcDhn5-OXP plants to be significantly more freezing tolerant than their untransformed counterparts.

### **Research on deacclimation physiology**

While autumnal cold acclimation is a gradual response (weeks to months) induced by short-days and cold temperatures, deacclimation proceeds much faster (hours to days) and is mainly driven by warm temperatures. Consequently, erratic temperature fluctuations, i.e., “unseasonal” spring-like conditions followed by a freeze, can render partially or fully deacclimated tissues vulnerable to freeze damage. Hence a plant both with high cold-acclimated hardiness and an ability to resist premature deacclimation under transient warm spells would be best positioned to escape winter cold injury. It may be reasonable to assume that plants with high midwinter hardiness would also exhibit a high degree of deacclimation resistance, and that a high cold acclimation capacity as well as deacclimation resistance both represent evolutionary responses to low minimum temperatures.

**Relationship between mid-winter hardiness and deacclimation kinetics:** We investigated this issue using nine azalea genotypes (both species and cultivars) that represented eight seed provenances, multiple USDA hardiness zones, and three habitat types: the southeastern lowlands, the Appalachian highlands, and the northeastern coastal region (Kalberer *et al.* 2007b). Buds from natural (field) cold acclimated plants were used to evaluate midwinter hardiness using a temperature controlled freeze-thaw protocol followed by visual estimation of freeze injury. To investigate whether there was an association between spring temperature fluctuations in their native habitats and deacclimation kinetics, we calculated the historic variation in temperature (Kalberer *et al.* 2007b) and the temperature range that each genotype was exposed to in its seed provenance location (NOAA 2002). Our study revealed two categories of azalea

genotypes: slow deacclimators (high deacclimation resistance) and fast deacclimators (low deacclimation resistance; Fig. 6), but there was no unequivocal relationship between proclivity to deacclimate and either the minimum temperature of habitats or the mid-winter hardiness. For example, *R. prunifolium* showed both low mid-winter hardiness and a high deacclimation resistance, while *R. canadense* had the highest midwinter hardiness among all genotypes investigated, but a low deacclimation resistance. Also, *R. canadense* (native to eastern Canada and the north-Atlantic USA, and freeze hardy to  $-28^{\circ}\text{C}$ ) in December deacclimated faster than the more sensitive Georgian species *R. prunifolium* (freeze hardy to  $-24.6^{\circ}\text{C}$ ; Kalberer *et al.* 2007a). The USDA hardiness zones for *R. canadense* are 3b to 7, and the minimum average temperature for this provenance is  $-10.9^{\circ}\text{C}$ , while that for the provenances of obtained *R. prunifolium* was warmer than  $-3^{\circ}\text{C}$ .



**Fig. 6.** Logarithmic linear models of bud freezing tolerance ( $LT_{50}$ ) as a function of days of deacclimation (DOD) for nine azalea genotypes. The upper curve represented six genotypes with high deacclimation resistance (slow deacclimators), and the lower curve represented three genotypes with high deacclimation resistance (fast deacclimators). (Adapted with permission from Kalberer *et al.* 2007a).

Though the data indicated that the degree of midwinter hardiness reflected the latitude and minimum temperatures of habitats, as expected, azaleas originating in cold climates and with high midwinter hardiness did not always have high deacclimation resistance, which is perhaps related to other climatic and developmental factors. These results suggest that deacclimation resistance and midwinter hardiness likely represent distinct “components” of winter hardiness and are perhaps inherited independently. These should therefore be evaluated separately when selecting and breeding for improved cold hardiness.

**Environmental and biological factors that promote high deacclimation resistance in plants:** Deacclimation resistance may be a function of climate variability

in the native habitat, particularly the variability during the late winter and early spring, rather than the low temperatures experienced *per se*. Logic then follows that plants growing under relatively stable conditions would experience little evolutionary pressure to develop deacclimation resistance to transient warming. Our azalea study indicated that on the whole, genotypes originating in the Appalachian highlands, which are prone to having significant fluctuations in temperature, have relatively high deacclimation resistance in comparison to those from coastal climates, where deacclimation might be less of a threat under the cool and relatively more stable temperatures typically experienced during coastal winter and early spring conditions. It may also be argued that genotypes with greater midwinter hardiness may experience less selective pressure for strong deacclimation resistance than do less hardy ones, because the former can safely lose a relatively large amount of acclimated hardiness before becoming vulnerable to cold injury.

Alternatively, high deacclimation resistance could be prevalent in plants with delayed spring development or deeper dormancy (Lang 1987), since resumption of growth can lead directly or indirectly to irreversible deacclimation (Leinonen *et al.* 1997). Ample evidence exists to show that spring growth and flower development are not conducive to the retention of hardiness, and that tissue hardiness is negatively correlated with flower development. Consistent with this notion, flower development of *R. prunifolium*, a slow deacclimator, was slower than for other native American deciduous azaleas; a slower transition to active growth could explain the longer maintenance of hardiness in these buds. The mechanism by which ontogenetic development modulates hardiness has been the subject of some speculation, but of relatively little research. Growth and development might have a negative effect on cold hardiness because higher cellular water content during cell expansion increases the probability of ice formation. This can render cells more susceptible to mechanical damage from extracellular ice, and increases the chance that ice will develop inside the living cells, which is always lethal. Alternatively, active growth might interfere with increasing deacclimation resistance by its competition for energy resources (Ogren 1997).

**Deacclimation response variability from early to late winter:** In another study using select azalea genotypes, we showed that fully cold acclimated buds of *R. viscosum* (var. *montanum*) deacclimated by ~40 % in February when exposed to deacclimating temperatures, but the same dose of warming in December resulted in only a 19% deacclimation (Kalberer *et al.* 2007a). It is noteworthy that at the December sampling, these buds were physiologically dormant, being in an endodormant state. This is the deepest state of dormancy in autumn, before buds have met their chilling requirement, i.e., exposure to a certain minimum amount of chilling in order to resume growth, measured in chill-hours. Once the chilling requirement is met, buds transition to

an ecodormant state, at which time the inability to grow is due exclusively to non-conducive environment, such as too cold a spring, or heat or drought. In the ecodormant state, plants are primed for growth as soon as they are exposed to a conducive, spring-like environment. In our study, buds had fulfilled their chilling requirement by the February sampling and were then less resistant to deacclimation than they were in December. The physiological rationale for why plants are less prone to deacclimation in the endodormant state is not yet well understood and deserves more research in the future. Nevertheless, the influence of dormancy status/bud-break on deacclimation behavior, both of which are regulated by temperature, can be impacted by unseasonal warming and has real world practical implications.

### **Research on light-stress and photoprotection strategies**

Overwintering evergreen species face a dilemma whereby their leaves may continue to receive light during cold winters when this energy cannot be efficiently processed due to their cold-induced slower photosynthetic biochemistry. Therefore, evergreens must possess mechanisms to manage and/or tolerate light stress during cold winters (Adams *et al.* 2004; Wang *et al.* 2008, 2009). Our laboratory conducted a comparative analysis of genes expressed in leaves collected in the summer (nonacclimated; LT50  $-7^{\circ}$  C) and winter (cold acclimated; LT50  $-53^{\circ}$  C) from field-grown *R. catawbiense* (Wei *et al.* 2005). We found lower expression of several photosynthesis-related genes (e.g., RuBisCO, a small subunit precursor, among others) in the winter leaves. This research also revealed that genes encoding a protein called ELIPs (Early light induced protein) were the most abundantly expressed of all the genes in winter leaves, while no ELIPs were detected in nonacclimated leaves (Wei *et al.* 2005). ELIPs are light stress induced proteins located in the thylakoid membranes of chloroplasts and belong to the chlorophyll *a/b*-binding protein family, with a wide distribution among plant species (Adamska 1997, Adamska *et al.* 1999). ELIPs may bind chlorophylls under high light stress and prevent the formation of free radicals and/or function in dissipation of excess light energy in the form of heat (Adams *et al.* 2004). ELIP accumulation, therefore, may constitute an adaptive response to winter conditions (cold and high light) in evergreens, and play a key role in the protection of photosynthetic apparatus from excess light (Zarter *et al.* 2006).

These ELIPs were later catalogued into seven distinct RcELIPs homologs (for *R. catawbiense* ELIPs; Peng *et al.* 2008b). Our later research revealed that the abundance of these seven RcELIPs changed incrementally from August to December in containerized plants ( $\sim$ 3-4 years-old) of two *Rhododendron* species, a less hardy *R. ponticum* (mid-winter leaf LT50  $\sim$ 20°C) and the super-hardy *R. catawbiense* (leaf LT50  $\sim$ 35°C; Wang *et al.* 2009). Interestingly, the rate of seasonal increase in ELIP

abundance for *R. catawbiense* was twice that for *R. ponticum* (Fig. 7), suggesting that *R. catawbiense* perhaps needs more efficient bolstering of photoprotection systems than does *R. ponticum*. This notion is curiously supported by the fact that *R. catawbiense* exhibits thermonasty while *R. ponticum* does not. Thermonasty refers to temperature-induced leaf movements (Nilsen 1987), a phenomenon where leaves droop and curl at freezing temperatures in winter (Fig. 7). One of the proposed benefits (among others) of thermonastic leaf movement is the avoidance of high light stress in the winter by reducing leaf exposure to light (Bao and Nilsen 1988), thereby facilitating the faster recovery of photosynthetic efficiency in spring (Russell *et al.* 2009). The lack of thermonasty in *R. ponticum* therefore suggests that this species is perhaps more tolerant of light stress in winter than is *R. catawbiense*. Our results on the seasonal patterns of photosynthetic efficiencies and photoinhibition in the two species support this hypothesis (Wang *et al.* 2009). This study also showed that leaves of both species had significantly higher activities of several antioxidant enzymes in the cold acclimated state; the primary role of these enzymes is to detoxify free radicals in plant cells which can accumulate in excess due to oxidative stress, including photo-oxidation.

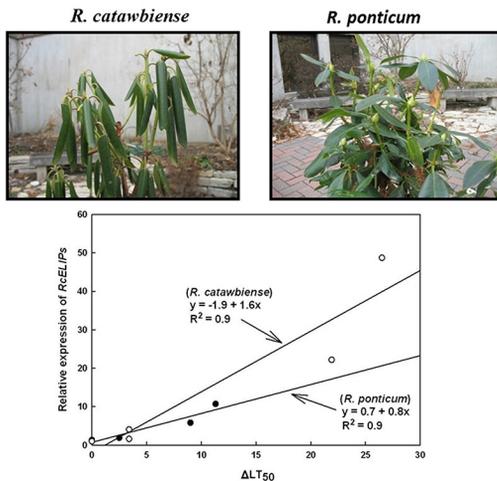


Fig. 7. The photographs in January (above panel) showing thermonastic leaf movements and lack thereof in *R. catawbiense* and *R. ponticum*, respectively. The lower graph shows the regressions of relative expression of RcELIPs on changes in leaf freezing tolerance ( $\Delta$ LT50), a measure of cold acclimation ability, for both *R. ponticum* and *R. catawbiense*. The relative expression of RcELIPs for each species in each month was calculated relative to their expression in August (taken as 1) and are based on the average of seven RcELIPs.  $\Delta$ LT50 are the absolute values of the LT50 difference between August and subsequent months till the December sampling (adapted with permission from Wang *et al.* 2009).

## Concluding remarks

Work in our lab has provided initial insights into the cellular and physiological mechanisms of freezing tolerance in *Rhododendron*. However, while *in vitro* studies with dehydrins suggest their importance in conferring freezing tolerance, more evidence for their *in vivo* role is warranted. In this regard, it would be useful to have a genetic transformation system for *Rhododendron* where dehydrin genes can be introduced and stably expressed. Such genetically modified plants could then be evaluated for their freezing tolerance in comparison to non-transformed controls. Also, rhododendrons, and most other plants too, contain a suite of dehydrin genes. This prompts the question as to whether some or all of these dehydrins work cooperatively to confer cryoprotection or freezing tolerance? Since, cold-acclimation is a multigenic complex response, it may be reasonable to hypothesize that two or more dehydrins cooperatively confer greater cryoprotection and freezing tolerance in an additive manner. Our work with *Rhododendron* F<sub>2</sub> progenies described above suggested that a few genes with strong additive effects were controlling variation in hardiness (Lim *et al.* 1998b).

Our deacclimation research has also suggested new research topics. For example, what determines the critical thermal dose (the combination of the extent and the duration of a warm spell) that triggers deacclimation in a given *Rhododendron* species or cultivar? We know that the dormancy status of the buds influences deacclimation behavior and that the bud break in the spring occurs only after meeting both the chilling requirement and the heat-unit requirement (release from ecodormancy). It is therefore important to include species-specific needs for the chilling requirement versus needed heat units to break ecodormancy, and both the magnitude and frequency of physiologically and regional/local relevant temperature fluctuations (rather than averages) in studies seeking to infer freezing tolerance responses under a changing climate.

Finally, more research is needed to better understand the physiological/biochemical mechanism of photoprotection by overwintering rhododendrons. In this regard, not only the performance of ELIPs, but also the utility of other changes such as the qualitative and quantitative fluctuations in xanthophyll carotenoid pigments, are being investigated in rhododendrons (J. Medeiros, pers. comm.). Accumulation of specific class of xanthophyll pigments has been previously associated with photoprotection in overwintering evergreens (Zarter *et al.* 2006), and they are believed to help dissipate excess light energy in the form of heat via non-photochemical quenching (Verhoeven *et al.* 1999, Adams *et al.* 2004). Work along this line of research is also underway in our laboratory.

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## Section: Physiological Ecology

### Chapter 3

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# Mini-review of *Rhododendron* Ecophysiology

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### Introduction

The species of *Rhododendron* serve as excellent models for studying the functional significance of adaptive traits. Such research is a primary underpinning of plant physiological ecology and, as a result, there is a long history of research on trait variation among species and its significance to the physiology and ecology of *Rhododendron*. This mini-review covers adaptive traits of photosynthesis, water relations, energy balance and nutrient relations. In particular, adaptive traits of leaves in response to habitat resource variation such as leaf size, trichome abundance, stomatal metrics, thermonastic leaf movements and idioblast cells are included. These diverse leaf adaptive traits occur in the context of the overriding constraints resulting from a highly safe against freezing embolisms but weakly efficient vascular system. Major ecological characteristics of *Rhododendron* species such as drought tolerance, cold adaptation, nutrient use efficiency and habitat preference are shown to be a syndrome that reflects the adaptive permutations of the vascular system.

### A primer of physiological ecology

The main goal of the scientific discipline of Plant Physiological Ecology is to explain or understand ecological patterns by employing research on physiological processes that underpin those ecological patterns. For example, in order to understand why plants in alpine habitats have different morphological and anatomical traits than similar species in lowland habitats, the climatic influences of elevation on physiological processes needs to be understood. Ultra-violet radiation (UV) is higher in alpine habitats and excessive UV radiation can inhibit physiological processes. Therefore, alpine plants have morphological, structural and chemical means of reducing the quantity of UV radiation that penetrates to important metabolic processes. In essence, physiological

ecology focuses on variation in physiological processes and the resulting functional traits of plants that control ecological patterns (Larcher 1995). Although there are many different physiological processes included in this discipline, photosynthesis, water relations, nutrient relations and energy relations are the most commonly studied (Nobel 1991). Therefore, this mini-review of *Rhododendron* physiological ecology will be focused on how these four physiological processes determine the performance (growth) of *Rhododendron* species in different habitat types.

Physiological ecology is particularly pertinent at this time because of the changes happening on the planet. Climatic warming will dramatically change the nature of local habitats and plants within their current ranges, because of their immobility, will either acclimate, adapt, or perish based on their physiological responses to those changes. Already, large-scale tree mortality occurring in conifer forests in western United States can be related to changes in water availability and its effect on physiological processes of those trees (Zhang *et al.* 2017). Moreover, the interface between climate change and expanding human habitation will inevitably create novel habitats that will test the physiological tolerances of plant species. Many *Rhododendron* species have been red-listed as endangered or threatened because of the nexus of climate change and human habitation (Ma *et al.* 2014). It is only through an understanding of the physiological ecology of plants that an effective conservation of plant based natural resources including *Rhododendron* species may be attained.

Research on the physiological ecology of *Rhododendron* has a long history and covers species across the northern hemisphere. My effort here is to summarize what that research has taught us about the physiological ecology of *Rhododendron* and its ecological implications. I will review what is known about the four main processes studied in physiological ecology and then summarize their combined effect on the ecology of *Rhododendron*. This mini-review covers general patterns, includes some important publications and provides a starting point for those interested in the subjects covered. This mini-review is meant to be an introduction to *Rhododendron* physiological ecology, and is not an exhaustive treatment.

## Photosynthesis

Photosynthesis has been studied in many species of *Rhododendron*, yet research has focused on the American *R. maximum* and the European *R. ferrugineanum*. Based on these studies, photosynthetic rates of evergreen *Rhododendron* species are characteristically low, compared with plants in general, because of low leaf nitrogen concentration and low stomatal conductance (Nilsen *et al.* 1988, Russell *et al.* 2009).

Net photosynthetic rates reach their highest rate during the first year after leaf formation and decrease slowly until leaf abscission (Nilsen 1992). In some *Rhododendron*

species, whole plant photosynthesis is dominated by the new flush each year and the older leaves serve primarily in nutrient conservation (Pornon and Lamaze 2007). The decrease in photosynthesis with age in evergreen *Rhododendron* is due to damage to chloroplasts by high light levels during cold winter conditions that adds up after each winter (Nilsen *et al.* 1988). This synergy of light intensity and cold conditions is accentuated in a temperate habitat where evergreen *Rhododendron* species grow under a deciduous canopy. In these habitats light level is low (because of the tree canopy) under the warmer conditions when leaves are made and mature; however, during the cold winter the canopy trees are leafless causing light intensity to increase for the evergreen *Rhododendron* leaves.

In comparison with leaves on the same plants in low light environments, plants in high light conditions have short leaf survivorship, their leaves are relatively small and photosynthesis is relatively high (Nilsen 1992). Light saturation of photosynthesis occurs at moderately low light intensity (400-700  $\text{mmol m}^{-2} \text{s}^{-1}$ ), even in environments with relatively high radiation (Nilsen 1992, Cai *et al.* 2014). Also, it only requires between 10 and 15  $\text{mmol m}^{-2} \text{s}^{-1}$  photosynthetic photon flux density (PPFD) to compensate for carbon lost by respiration. Therefore, photosynthesis in *Rhododendron* is adapted to relatively low radiation, and *Rhododendron* species acclimate to high radiation by reducing leaf size and shortening leaf longevity instead of dramatically changing the light response patterns of photosynthesis (Bao and Nilsen 1988, Lipscomb and Nilsen 1990).

The light reactions of photosynthesis reside on internal membranes of the chloroplast and are particularly sensitive to damage by excessive radiation. Snow cover can prevent photo-inhibition by high light (Neuner *et al.* 1999) for *Rhododendron* species in alpine areas, but in habitats with less snow cover, evergreen leaves must either avoid the high light or have physiological photo-protection mechanisms. There are small molecular weight proteins in the chloroplast called Early Light Induced Proteins (ELIP), which are capable of preventing some of the damage by excessive radiation during the excessively cold winter temperatures. ELIPs have been shown to increase in concentration during cold conditions in some *Rhododendron* taxa (Peng *et al.* 2008). High light during cold conditions can cause an increase in reactive oxygen molecules such as ozone, hydrogen peroxide and singlet oxygen. Plants can remove those reactive oxygen molecules using enzymes in the chloroplast, such as with peroxidases and catalases, and both these compounds increase in abundance during cold adaptation in some *Rhododendron* species (Wang *et al.* 2009). Therefore, temperate evergreen *Rhododendron* species have physiological capacity to protect their leaves from damage by high light in the winter. However, the strength of the physiological acclimation to cold varies significantly among species and may have important implications to the distribution of species

(Wang et al. 2009). Those species that have evolved physiological tolerance are able to have a wider range and have greater tolerance to environmental variation.

If the physiological ability to compensate for the cellular disruptions caused by excessive radiation is only partially effective, then a mechanism of avoiding the excessive radiation is important. The negative influence of high light during the winter can be avoided by dropping leaves in the winter (being deciduous) or moving the evergreen leaves away from the light source when it is cold by lowering them or rolling them up (thermonastic leaf movements). Many evergreen species of *Rhododendron* that inhabit temperate forest environments have thermonastic leaf movements (Figure 1). In fact, there is a very good correlation between the strength of cold tolerance and the amount of thermonastic leaf movement (Nilsen 1991, Nilsen and Tolbert 1993). Leaves droop (curl) and roll in response to low air temperature during the winter. Leaf curling is primarily determined by petiole turgor pressure (Nilsen 1987) while rolling is primarily determined by leaf lamina temperature (Nilsen *et al.* 2014). When leaf curling is prevented during the winter, quantum yield (increase of photosynthesis as light intensity increases) of photosynthesis is inhibited (Nilsen and Bao 1988), and light saturated photosynthesis decreases the following spring (Nilsen 1992, Russell and Nilsen 2009). Understory plants can gain most of their annual carbon gain during the early spring before canopy trees leaf out. Therefore, thermonastic leaf movements protect *Rhododendron* leaf photosynthesis during the coldest conditions in the winter

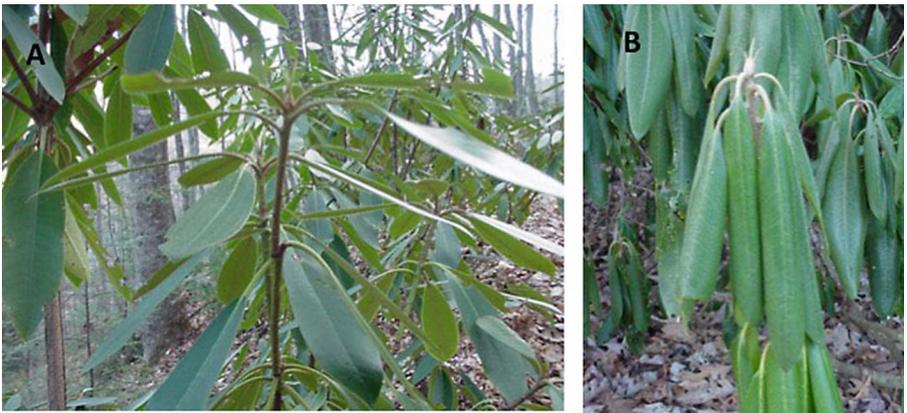


Figure 1: Images of *Rhododendron maximum* leaves in the understory of a southern deciduous forest in Virginia, USA. A) Leaf position during warm periods in the winter. B) Leaf position when the air temperature is below 2°C. The position of these leaves is a consequence of thermonastic leaf movements.

so that leaves can take advantage of the early spring light (Russell and Nilsen 2009).

The dynamics of photosynthesis for evergreen *Rhododendron* species in tropical environments ought to be very different from those in temperate environments. Very little is known about photosynthesis of tropical *Rhododendron* species. My personal measurements of photosynthesis of three tropical vireya species (growing in a greenhouse) suggest that maximum rates of photosynthesis are similar to those for temperate species. I am unaware of any published research at this time on photosynthesis of tropical *Rhododendron* species.

### **Water relations**

Plant traits associated with water accumulation, transport, distribution and loss have important implications for plant physiological ecology. Those hydrological traits can determine plant habitat distribution, the morphology of plant parts and their anatomy. In fact, several anatomical and morphological traits are considered indicators of drought tolerance (Nilsen and Orcutt 1996). One way to characterize water relations functional traits is to separate liquid water flow (hydraulic traits) from vapor based traits (transpiration). Although we separate liquid flow from vapor flow in this mini-review, we understand that these traits are intimately linked because they are arranged in a series; liquid water flows from the roots to leaves and then evaporates out stomata to the atmosphere.

The great majority of *Rhododendron* species have stomata only on the underside of the leaf (hypostomatous). However, one species (*R. saxifragoides*) has stomata on both leaf surfaces (Nilsen and Tulyananda 2015), which is maintained in 50% of the plants when hybridized with a hypostomatous species (Nilsen 2011). Stomatal density varies from 50 to 700 per mm<sup>2</sup> among species (Nilsen *et al.* 2014) and stomatal size can vary by up to 50% among species. Potential transpiration from the leaf is directly related to the product of stomatal density and the square of stomatal size, i.e., the Stomatal Pore Index (SPI = density x size<sup>2</sup>). Therefore, transpiration can vary greatly among species due to the variation in both density and size. However, in most plant species, stomatal density is negatively correlated with stomatal size, which reduces the variation in SPI, and this negative correlation holds for *Rhododendron* species. Nevertheless, in comparison to many other plant species, *Rhododendron* stomatal size is significantly smaller at any stomatal density (Nilsen *et al.* 2014). Thus, the SPI of *Rhododendron* species suggests low potential transpiration and greater water conservation. Stomatal pore index can increase with an increase in elevation, suggesting higher whole plant water use. However, leaf area decreases with increasing elevation, and nutrition increases, resulting in an increase in water use efficiency at high elevations compared with that at low elevations (Taneda *et al.* 2016).

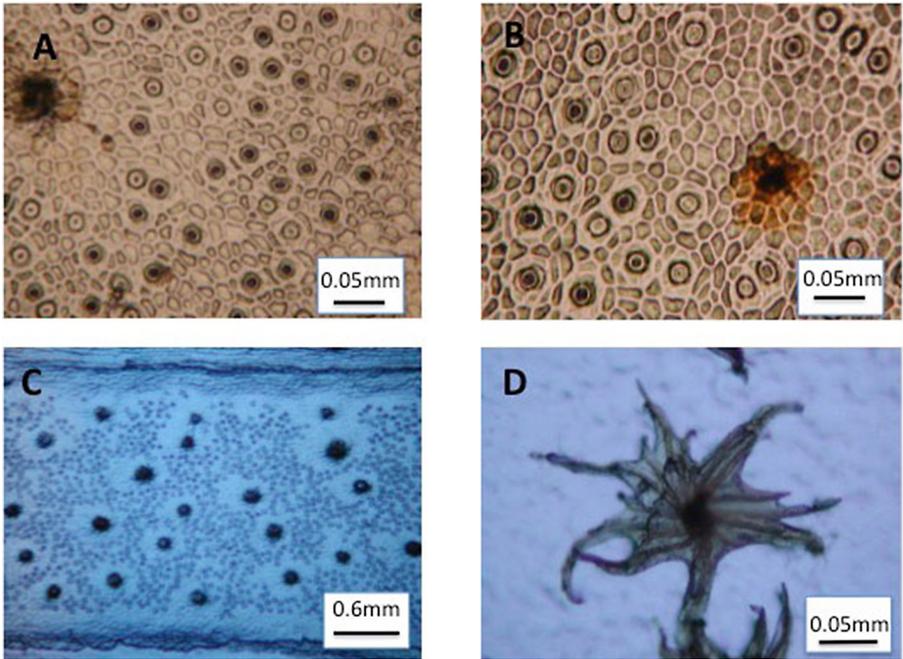


Figure 2: Light microscopic images of scales and stomata on *Rhododendron* species leaves. A) Stomata and one peltate scale on the adaxial surface of a *R. saxifragoides* leaf. B) Stomata and one peltate scale on the abaxial surface of a *R. saxifragoides* leaf. C) Scales and stomata on the abaxial leaf surface of *R. stenophyllum*. Note the region around the scale bases that are devoid of stomata. D) Dendroid scale from *R. bryophyllum*.

Most measurements of *Rhododendron* transpiration, particularly for evergreen species, are low when compared to other plant species (Lipp and Nilsen 1997). This low transpiration may be partly a consequence of low SPI, interference by epidermal hairs and scales, anatomical traits of leaves or the sums of any or all these processes. Abaxial hairs and scales are morphological traits on *Rhododendron* leaves that vary in shape, height and density dramatically among species and can have a significant effect on water conservation (Nilsen *et al.* 2014). The abundance of scales affects water conservation because of the amount of epidermal surface they occupy (Figure 2). The greater the epidermal surface occupied by scales and the stomatal free zone around the scales, the greater will be the water conservation (Nilsen *et al.* 2014, Nilsen and Tulananda 2015).

Water conservation is also determined by anatomical traits. Leaf succulence reaches its highest amount in tropical species of *Rhododendron*, possibly because many are

epiphytic (Tulananda 2016). Idioblasts, which are large cells filled with water near the top epidermis (Nilsen and Scheckler 2003), enhance succulence in relatively thin leaves (Figure 3). Moreover, the larger the number of idioblasts in the leaf, the greater will a plant's ability to maintain turgor at low leaf water content (Tulyananda and Nilsen 2017). Specialized anatomical traits have evolved for drought tolerance in tropical *Rhododendron*, with idioblasts confined to species of vireya (section *Schistanthe*), which is the largest section in genus *Rhododendron*. Every member of section *Schistanthe* that I have checked (150+ species) have idioblasts, except for *R. saxifragoides*. At this time I have found only one non-vireya species that may have well-developed idioblasts (*R. camelifolia*). However, some tropical species outside of vireyas do have a hypodermis

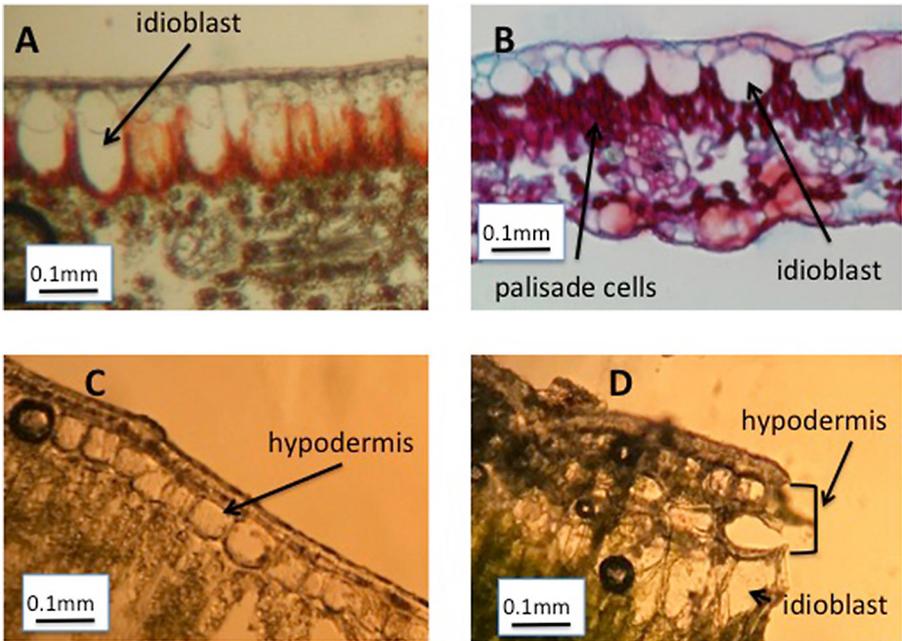


Figure 3: Images of idioblasts in leaves of tropical *Rhododendron* species. A) Free hand cross section of a *R. brookeanum* leaf showing a continuous sheet of idioblasts just below the adaxial epidermis and extending past the bottom of the palisades cells (shown in red); B) Microtome section stained with saffranin of a *R. celebicum* leaf showing both abaxial and adaxial epidermis and idioblasts below the adaxial epidermis but not extending into the palisades cell layer; C) Hand cross section of a *R. nutallii* leaf showing the hypodermis common for leaves on species in subsection *Maddenia*, but no idioblasts; D) Free hand cross section of *R. pudorinum* leaf showing the presence of both a hypodermis and idioblasts.

(Figure 3) and some vireyas such as *R. pudorinum* have both a hypodermis and idioblasts. Adaxial idioblasts may have evolved from a hypodermis in vireyas, but this evolutionary trajectory is unconfirmed.

Even though drought tolerance mechanisms are evident in the structure and function of *Rhododendron* leaves, most *Rhododendron* species inhabit relatively humid and wet habitats. The vascular anatomy of *Rhododendron* stems and its effects on hydraulic flow is a main reason why *Rhododendron* species have drought tolerant leaf traits and low transpiration, yet are restricted to moist habitats. Vessels in *Rhododendron* stems (Figure 4) are particularly narrow compared to many other plant species (Tulyananda 2016, Cordero and Nilsen 2002, Lipp and Nilsen 1997, Noshiro 1995). Narrow vessels increase resistance for water flow in stems, which constrains the amount of water flow the stems can transport to leaves. Given the constraint on hydraulic flow, it is likely that *Rhododendron* leaf water content will decrease quickly if too much transpiration occurs, resulting in water stress. Thus, *Rhododendron* species characteristically have low transpiration rates, stomatal closure early in the day and leaf water conservation features when growing in habitats that have high water evaporative demand (Lipp and Nilsen 1997).

Because the water transport vessels in *Rhododendron* stems are particularly narrow, they are also fairly sensitive to drought induced water flow failure caused by embolisms in the vessels (Lipp and Nilsen 1997, Cordero and Nilsen 2002). Tension caused by excessive evaporative demand relative to water flow capacity in the stem can create embolisms, which can completely block water flow and cause tissue death. Therefore, leaf transpiration must be kept relatively low to avoid stem, and possible plant, death. However, narrow vessels can be beneficial, as they are an important defense against freezing damage on hydraulic flow in temperate *Rhododendron* species.

During a freeze, air in stem water can come out of solution and cause bubbles (embolisms) in the vessels. These freeze-thaw induced embolisms can cause 100% blockage of water flow in the winter. Many temperate forest trees must construct new vessels in the spring before creating new leaves because of this freeze-induced blockage of water flow in older vessels that were made during the previous growing season. As mentioned above, evergreen *Rhododendron* shrubs in temperate habitats gain a large portion of their annual carbon gain in the early spring. Therefore, building new vessels in the spring would greatly reduce this important carbon gain in *Rhododendron* species and potentially result in carbon starvation. However, freeze induced embolisms can be prevented (bubbles are less likely to coalesce into a large bubble in a narrow vessel during a freeze-thaw event) or quickly repaired if vessels are narrow (Medeiros and Pockman 2014). In accordance, vessels of evergreen *Rhododendron* species that inhabit cold climates are narrower than those that inhabit warmer climates (Cordero and

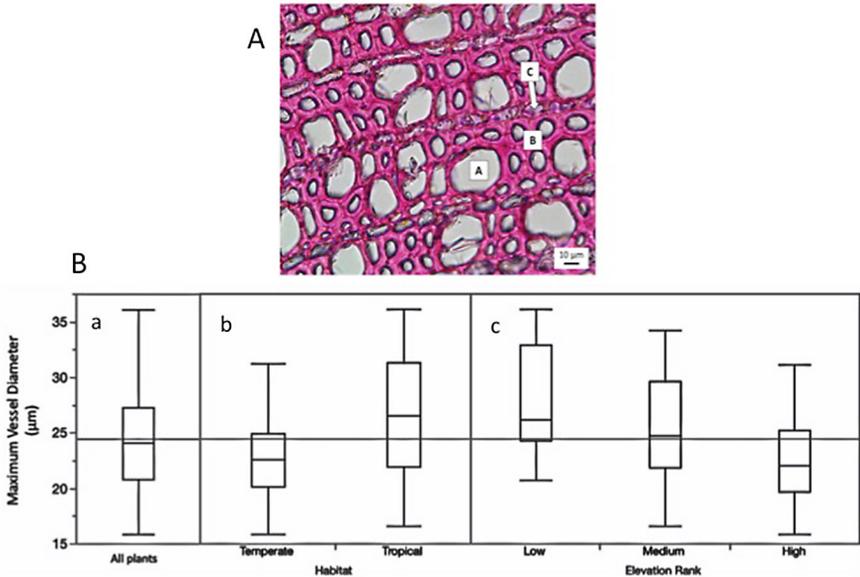


Figure 4: Characteristics of a *Rhododendron* vascular system. A) Cross section of wood from a stem of *R. jasminiflorum* stained with safranin. A = a vessel element, B = a fiber, C = a parenchyma cell; B) Results from measuring mean vessel diameter across 70 *Rhododendron* species from temperate and tropical habitats. All species were growing at the *Rhododendron* Species Foundation and Botanical Garden, Federal Way, Washington, USA. a) mean vessel diameter for all sampled species, b) mean vessel diameters for tropical or temperate species, c) mean vessel diameter for species whose mean native habitat elevations are low (0 – 1200 m), medium (1201 – 2400 m), or high (>2400 m).

Nilsen 2002). Thus, the narrow vessels of evergreen *Rhododendron* stems are an effective protective measure against freeze-induced embolisms (Lipp and Nilsen 1997, Cordero and Nilsen 2002).

Given that narrow vessels protect against freeze-induced embolism yet constrain water flow, this trait may be maladaptive for tropical species of *Rhododendron* that do not experience any freezes. Having narrow vessels is considered a safety trait, while having relatively high hydraulic flow is considered an efficiency trait. Because both the safety and efficiency of stem water flow is dependent upon vessel traits, a trade-off results. Narrow vessels at a low density result in high safety while dense, larger diameter vessels lead to high efficiency. A recent comparison of the twig wood from temperate and tropical species of *Rhododendron* has shown that tropical species have higher efficiency and lower safety that do temperate species (Tulyananda and Nilsen 2017). Moreover, stem safety increases as the elevation of the native range increases for both temperate

and tropical *Rhododendron* species. However, the increase in efficiency of tropical *Rhododendron* stems is small, and water flow constraint still dominates the hydraulic flow of these species. Leaves on tropical species also have anatomical traits reflective of greater drought tolerance than their temperate counterparts (Tulyananda 2016). In fact, leaf succulence traits are much more developed in tropical *Rhododendron* species when compared to temperate species. Therefore, as shown by their stem anatomy, water conservation is still important for both temperate and tropical *Rhododendron* species.

### **Nutrient relations**

*Rhododendron* species are commonly found in habitats with acidic soils that have relatively low nutrient concentrations, particularly for nitrogen. The ammonium ion is the most available form of soil nitrogen in low nutrient acidic soils, and so most *Rhododendron* species preferentially absorb ammonium ions rather than nitrate ions. The low nutrient content of leaves results in a recalcitrant leaf litter with a relatively high carbon to nitrogen ratio, which decomposes relatively slowly in streams and soil. The result is a negative feed back loop in which increases in the biomass of *Rhododendron* results in a further decrease in soil nutrition, particularly for nitrogen. Thus, if a major herbivore of *Rhododendron* (such as white tailed deer) is removed from an ecosystem, the shrub abundance increases and the soil nutrition decreases (Zamin and Grogan 2013).

Many plant species have a symbiotic relationship with soil fungi called mycorrhizae. Types of mycorrhizae are classified based on the type of fungus and the way the fungus joins the roots of the associated plant. Some fungi are exclusively on the outside of roots (ectomycorrhizae) while other fungi integrate into the root cells (endomycorrhizae). Another class of mycorrhizal fungi only infects species of the plant family Ericaceae (ericoid mycorrhizae), which is the case for *Rhododendron* species. Ericoid mycorrhizae are endomycorrhizae whose mycelia (fungal body) extend out into the very top surfaces of the soil and litter. Therefore, ericoid mycorrhizae can absorb nutrients immediately as they are decomposed by the fungi from the litter, which may prevent roots and mycorrhizae of other species from getting those nutrients. Also, nitrogen is mineralized as ammonium ions and later converted to nitrite and eventually nitrate by soil bacteria. Thus, ericoid mycorrhizae are likely capable of absorbing excess ammonium ions. When the excess of soil ammonium ions is taken in by the mycorrhizae, some of the absorbed nitrogen can diffuse into the *Rhododendron* plant and enhance the host plant's nutrient accumulation.

Litter (dead leaves, flowers and stems) of *Rhododendron* species tends to be nutrient poor, and can also release compounds into the soil that have negative effects on soil nutrition. As a result, the soil nutrition level under dense colonies of *Rhododendron*

plants can become significantly lower than the level in soils in the surrounding area (Horton *et al.* 2009). Moreover, polyphenols that are released from the decomposing litter of *Rhododendron* plants can inhibit nitrogen cycling bacteria, thereby limiting the conversion of ammonium to nitrate (Wurtzburger *et al.* 2007), which in turn will increase the relative abundance of ammonium ions for rhododendrons. It should be noted that in *Rhododendron*, a high proportion of nutrients are reabsorbed into the plant body before leaves are lost to litter. This indicates that although nutrient levels, and particularly nitrogen, are generally low in *Rhododendron* leaves, nutrient use efficiency is good because of their strong retention of nutrients.

### **Energy balance relationships**

The temperature of leaves is intimately related to the leaf energy balance. Under conditions when the energy input is greater than the output, leaf temperature increases. The most important energy inputs to *Rhododendron* leaves are solar radiation and infrared radiation. The amount of solar radiation hitting the leaf depends upon the exposure of the leaf to the sunlight. The amount of infrared radiation coming to the leaf depends upon the relative temperature of the environment around the leaf. The higher the temperature of the objects around the leaf, the higher the infrared input to the leaf. The most significant outputs of energy from a *Rhododendron* leaf are latent heat exchange from water evaporating off the leaf through their stomata and conductive heat loss directly from the leaf surface to the turbulent air around the leaf. The magnitude of latent heat exchange is dependent upon transpiration, which on a leaf area basis is relatively low for *Rhododendron* leaves compared to other plant species. The amount of conductive energy loss from a *Rhododendron* leaf is inversely dependent upon the length of the leaf in the direction of the wind (Nobel 1991). Therefore, the larger the leaf, the lower will be its conductive heat loss. As a result of the balance between inputs and outputs of energy to a *Rhododendron* leaf, there is a tendency for larger *Rhododendron* leaves to heat up in regions of higher energy more than would those plants with smaller leaves. This is one reason why leaf area is smaller for plants of any species when growing in higher light sites when compared to their growing in lower light sites (Nilsen 1986).

*Rhododendron* that inhabit forests are generally bathed in low radiation intensity due to the forest canopy. However, gaps in the forest canopy can result in patches of high radiation that penetrate down into the understory where the *Rhododendron* reside (sunflecks). Leaf temperature rapidly increases when leaves on *Rhododendron* in an understory experience a sunfleck. I have measured up to a 15°C increase in leaf temperature over air temperature for *R. maximum* leaves in a sunfleck. This elevated leaf temperature was not detrimental to the *R. maximum* leaves and in fact, the temperature optimum for photosynthesis was that of the elevated leaf temperature experiencing a

sunfleck.

Ultraviolet radiation (UV) is part of the total radiation impinging on the leaf surface. High-energy UV is a relatively small component of the total solar energy hitting a leaf because most UV is removed by ozone in the atmosphere. Thus, UV has an insignificant effect on leaf energy balance, yet UV can have a detrimental effect on metabolism and genetics because nucleic acids and proteins are sensitive to UV. The atmosphere thins near the poles and is thinner at high elevation sites, which makes the potential impact of UV on plant metabolism greater closer to the poles and at higher elevations. Therefore, adaptations for reducing UV radiation are more likely to occur in *Rhododendron* species in northern habitats and at high elevations.

Mechanisms that plants use to limit UV exposure include both structural and chemical characteristics. For example, a thick layer of wax on the top leaf surface and a multiple layer epidermis can reduce the intensity of UV in the leaf region that performs most photosynthesis (Palisades and spongy mesophyll). In addition, there are several chemicals that absorb UV radiation, such as anthocyanins. A high concentration of UV absorbing molecules in the top epidermis can significantly reduce UV radiation load inside a leaf. Another important UV protection mechanism is an ability to repair the damage created by UV exposure. Plants have a receptor for UV type B that induces the synthesis of both DNA repair enzymes that repair damage to nucleic acids from UV exposure and of antioxidant enzymes to eliminate reactive oxygen molecules from the cells.

Few studies have been performed on the consequences of UV exposure in *Rhododendron* leaves, but it is likely that high elevation species of *Rhododendron* ought to have more effective mechanisms in both reducing UV impacts and repairing UV exposure damage on the plant's metabolism. For example, the photochemical reflectance index (a measure of the proportion of UV reflected) is relatively high for *R. ferrugineum* at high elevation sites compared with other plant species at the same site (Filella and Peñuelas 1999). Evergreen leaves like those of *R. ferrugineum* in alpine habitats benefit from UV protection mechanisms. Also, methods of UV radiation protection generally increase as leaves age due to changes in their cuticular thickness and leaf structure that may not be related to quantity of UV screening chemicals (Rhuland and Day 1996). Thus, there is a complex relationship between habitat, leaf longevity, leaf age, leaf structure, leaf chemical composition and repair mechanisms that determine the relative tolerance of *Rhododendron* leaves to UV exposure.

## General Implications for *Rhododendron* physiological ecology

The functional traits for *Rhododendron* considered in this mini-review can be melded into a general syndrome that governs the way *Rhododendron* species interact both with their environment and with other species. Wood anatomy is central to *Rhododendron* functional traits and governs *Rhododendron* distributions and interactions with other species.

Restricted stem water flow due to narrow vessels limits productivity, promotes leaf longevity and predisposes plants to a high resource use efficiency. However, wood anatomy can also constrain species to moist habitats in order to minimize the likelihood of drought. Although wood anatomy constrains *Rhododendron* species to a low productivity, *Rhododendron* species can be very competitive because of their high resource use efficiency and their negative impact on availability of resources by other species. Thickets of *Rhododendron* can create areas of low light, poor nutrients and limited water, i.e., a habitat where many other species cannot survive (Beier *et al.* 2005). Moreover, the number of *Rhododendron* species that are trees speaks to the ability of *Rhododendron* leaf and root adaptations that increase xylem flow enough to support the water needs of a canopy tree.

Wood anatomical features also promote the survival of *Rhododendron* at high elevation and latitude because of their protection against damage by freezing. A major variation in leaf traits such as size, anatomy, movement and surface appendages has allowed *Rhododendron* species to diversify into many different habitat types. Therefore, even though wood anatomy might have constrained *Rhododendron* species to a limited number of suitable habitats, adaptations in leaf functional traits have compensated and have allowed *Rhododendron* species to proliferate and succeed in a wide diversity of habitats. Moreover, it may be that the ability of *Rhododendron* to adapt to a diversity of habitats has resulted in a wide diversity in its flower structure due to the variation in pollinator populations among locations. In summary, the wood anatomy of *Rhododendron* is the underlying attribute that has led to high resource use efficiency, a strong competitive ability, a wide diversity of leaf functional traits and the many characteristics of flowers..

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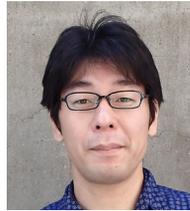
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# **Altitudinal Trends of Efficiency and Stability of Water Transport in the Stems and Leaves of Nepalese *Rhododendron* Species, Based on Morphological Traits**

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## **1. Introduction**

### **1-1. *Geographical distribution of Nepalese Rhododendron***

Over 30 *Rhododendron* species occur in Nepal (Noshiro 1997, Yoshida 2005), where climate ranges from humid in the eastern part of the country to xeric in its western part, and with altitude from tropical to alpine tundra (Singh and Singh 1987). Growing abundantly in many places, rhododendrons are familiar to Nepalese people, and the red color of *R. arboreum*, the national flower of Nepal, is used as the background color of the Nepalese national flag (see [http://www.easynepalotyping.com/nepali\\_flag](http://www.easynepalotyping.com/nepali_flag)). Nepalese *Rhododendron* species occur more densely in eastern mesic regions and range from 1500 (4950 ft) to 5000 m (16,500 ft) in altitude (Noshiro 1997). In the eastern region, the dominant vegetation changes distinctively with altitude: evergreen broad-leaved tree forests dominated by *Quercus* species between 1500 and 2500 m; winter deciduous tree forest (*Acer-Magnolia* forest) between 2500 and 3000 m; mixed forest of evergreen conifers and deciduous broad-leaved trees (*Abies-Betula* forest) between 3000 m and 4000 m; timber line around 3800–4000 m; alpine mat vegetation above 4200 m (Singh and Singh 1987, Noshiro 1997). The Nepalese rhododendron species occur at all of

these vegetation zones as canopy trees, understory shrubs, and as prostrate alpine shrubs at open sites (Figure 1a–c, de Milleville 2001, Noshiro 1997, Yoshida 2005). Each species has a wide altitudinal habitat range, between 500 and 1000 m. Notably, some species, such as *Rhododendron arboreum* and *R. lepidotum*, occur over 2000 m altitudinal difference (Noshiro 1997).

The diversifications of the Nepalese *Rhododendron* species and habitats are associated with adaptations to different altitudinal environments. As altitude increases, air temperature decreases by 5–6°C (9–11° F) per 1000 m, growing period shortens, and soil temperature decreases, but precipitation and relative humidity increase (Jump *et al.* 2009, Körner 2003). Generally, plant growth is sensitive to changes in temperature and moisture, and plants can change their morphological and physiological properties to acclimate to respective altitudinal conditions (e.g., leaf nitrogen content, stomatal density, shoot to root ratio (Körner 2003); timing of bud break (Vitasse *et al.* 2009); acquirement of freezing resistance (Ishizuka *et al.* 2015)). Plant water use is also an important factor in altitudinal acclimation (Mayr and Charra-Vaskou 2007, Petit *et al.* 2011, Smith *et al.* 2003). However, few studies have addressed altitudinal change in the hydraulic properties of plants. Because Nepalese *Rhododendron* species are found in a wide range of



**Fig. 1.** Study sites and vegetation of *Rhododendron* trees and shrubs at different altitudes.. (a) Forest at ca. 2600 m where *Rhododendron arboreum* subsp. *cinnamomeum* over 10 m tall dominated. Arrows: two people walking along the trail. (b) Subtree forest comprising *R. arboreum*, *R. campanulatum*, *R. hodgisonii*, and *R. thomsonii* with 3-4 m high canopies at ca. 3350 m (c) Slope in alpine tundra covered with *R. anthopogon*, *R. lepidotum*, and *R. setotum* shrubs at ca. 4100 m, and (d) Locations of the study areas in eastern Nepal, Rolwaling and Jaljale.

altitude, they likely show distinctively different water use and water transport properties to adapt to the different soil moisture and atmospheric vapor deficit conditions across elevation.

### 1-2. *Plant water transport and adaptation to high altitude*

Water is a vital resource, which is consumed during plant growth as transpiration. During photosynthesis, CO<sub>2</sub> is absorbed from the atmosphere by mesophyll tissues via stomata, and simultaneously water vapor is transpired from leaves (Jones 2013). To assimilate 1 g of carbohydrate, angiosperm trees lose 200–300 g of water from their leaves as transpiration (Fitter and Hay 2002). Transpiration rate from a leaf is determined by the vapor-pressure difference between the atmosphere and the internal space inside a leaf (VPD, or transpiration demand), and by the extent of stomatal opening (stomatal conductance, *g<sub>s</sub>*). To compensate for the transpired water and maintain photosynthesis, efficient water transport from the soil to the leaves is required (Jones 2013). Water flow through a plant body is driven by surface tension, generated by evaporation from the surfaces of cell walls inside the leaf internal space. The surface tension pulls on the water column, whose water molecules are connected to each other by hydrogen bonds (cohesion-tension theory, Tyree and Zimmermann 2002). Water moves through vessels or tracheids in the xylem for most of the long distance transport in the plant body, while water passes through parenchyma tissues, including pathways in the cell walls, cytoplasm, and cell membranes for short distances in the roots and leaves. For xylem water transport, the number and diameter of xylem conduits are pivotal traits, because water conductive capacity is proportional to the fourth power of conduit diameter (Tyree and Zimmermann 2002). Due to xylem tension, water in a conduit is in a meta-stable state. If gas bubbles enter into the lumen of a water-filled conduit (an event called xylem cavitation), dysfunction of water transport happens due to a breakage of the connections between the water molecules (Tyree and Zimmermann 2002) [Editors note: xylem embolism is also discussed in this volume by Nilsen]. By contrast, passage of water through cell membranes (in a leaf, for example) is a challenge, and the permeability of the cell membrane is controlled via gene expression and activation by phosphorylation of aquaporins, or cell membrane proteins that provide water channels, in response to the environmental stimuli (reviewed in Maurel *et al.* 2015).

There are two plausible responses of hydraulic properties of leaves and stems to different altitudes. At high altitudinal habitat, low air temperature and high relative humidity greatly reduce transpiration demand, while high stomatal conductance has been reported in plants growing at high altitude (Körner 2003). Therefore, water transport systems with tissues that are less conductive and less

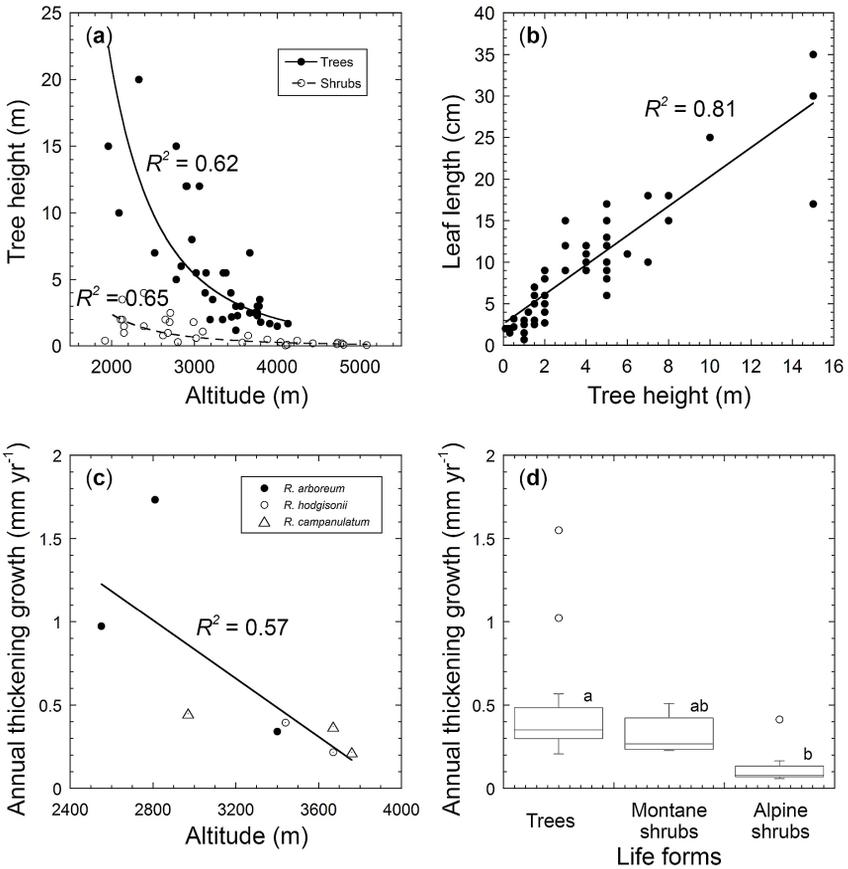
vulnerable to xylem cavitation may be sufficient to compensate for water loss, and be adaptive, at high altitude. On the other hand, the conductive capacity of plant tissues decreases with decreasing temperature. Due to higher viscosity of water at lower temperatures, the conductive capacity of xylem transport can be reduced by 20% as a consequence of decreasing the temperature by 10°C. For water transport through cell membranes, additionally, the conductive capacity decreases by 50 to 70% as the temperature decreases by 10°C (Ionenko *et al.* 2010). Under clear and less windy conditions, leaf temperature and transpiration demand can rapidly raise (Larcher *et al.* 2010). In this situation, strong xylem tension is induced in xylem sap, resulting in catastrophic xylem cavitation and plant death, unless water supply is kept up with transpiration (Tyree and Zimmermann 2002). Thus, *Rhododendron* trees growing at high altitude may have stems and leaves which are both conductive and resistant to xylem cavitation, but at a high carbon cost.

This review aimed to reveal altitudinal trends in the efficiency and stability of water transport in the leaves and stems of rhododendrons growing in eastern Nepal (Jaljala Himal and Rolwaling, Figure 1d). We re-visited our previous studies of anatomy and hydraulic properties of stems and leaves of Nepalese *Rhododendron* and evaluated their hydraulic efficiency and stability using the associated morphological traits that the previous studies have indicated. For stem traits, we used data about interspecific differences among 26 *Rhododendron* species (Noshiro *et al.* 1995), intraspecific differences within *R. arboreum* (Noshiro and Suzuki 1995) and ontogenetic analyses of the stem xylem of 15 species (Noshiro and Suzuki 2001). These data were collected from one to three cores of stem xylem at breast height or at the stem base of aboveground parts, if plants were short. Leaf traits were measured on five *Rhododendron* species growing between 2750 m and 4700 m in altitude (Taneda *et al.* 2016). The leaves were collected from the highest and lowest limits of the altitudinal distribution of each species. The present review categorized the *Rhododendron* species into three groups: trees, montane shrubs, and alpine shrubs. Montane and alpine shrubs are defined as *Rhododendron* shrubs growing below and above 3800 m in altitude, respectively. We also add new data, evaluating the hydraulic properties among the three life forms. Based on these results, we assessed the following questions: (1) what plant traits change with altitude?; (2) Is efficiency and stability of stem water transport high in *Rhododendron* species growing at high altitude?; (3) Is efficiency and stability of leaf water transport high in *Rhododendron* species growing at high altitude?; (4) What is the inter-specific difference in water conductive capacity through whole plant?

## 2. Altitudinal changes in species' morphology

Plant height changed with altitude for 26 *Rhododendron* species (Fig. 2a). For tree species, plant height sharply decreased with increasing altitude, to about 2 m around timber line. As shown in Fig. 1, notably, the tree *Rhododendron* species constituted the canopy of the forest. Decreased plant height with altitude was also found in other canopy species besides rhododendrons (Singh and Singh 1987). *R. arboreum* trees that occurred from 1500 m to 4000 m decreased their height with increasing altitude (Noshiro and Suzuki 1995). Likewise, plant height decreased with increasing altitude in *Rhododendron* shrubs. The change in plant height is probably due to adaptation to physical load by wind and snow, because the canopy height was nearly uniform (Smith et al. 2003). Notably, leaf length increased with increasing tree height (Fig. 2b). Among 51 *Rhododendron* species found in eastern Nepal, Tibet and Bhutan (Yoshida 2005), the relationship between leaf length and plant height was very tight. Leaf size is related to branching pattern (Westoby et al. 2002). Large leaves are found in less branched trees, and these are often fast-growing or early-successional species. Thus, this tight correlation implies that the plant growth pattern is related to plant height in Nepalese rhododendrons.

Another plant trait changing with altitude is the rate of stem radial growth. The annual radial growth of rhododendrons estimated from Noshiro and Suzuki (2001) decreased with increasing altitude of their habitat (Fig. 2c). Cambial activity was hindered by low air temperature, which limited developmental activity and the duration of stem thickening at high altitudes (Li et al. 2016, Petit et al. 2011). Between species, the annual radial growth of alpine shrub species was markedly lower than that of species growing at lower altitudes (Fig. 2d). An extremely small annual growth rate of 0.1 to 0.3 mm/yr was also observed in *R. aganniphum*, occurring at 4300-4600 m on the Tibetan Plateau (Li et al. 2016). A thickened stem xylem reinforces its mechanical strength and enhances its water conductive capacity, leading to an increased leaf forage area and carbon gain (Taneda and Tateno 2004). The extremely small annual radial growth of alpine rhododendron shrubs places them at increased risk of growth inhibition (Petit et al. 2011). The cambial activity of *R. aganniphum* is only limited at a minimum air temperature of about 2°C. This minimal growth threshold temperature of alpine rhododendrons is lower than that of evergreen conifers occurring around the timberline (4–5°C; Boulouf et al. 2012). The narrow annual growth rings of alpine rhododendrons have been suggested to be the result of adaptation to severe alpine climate conditions (Li et al. 2016).



**Fig. 2.** Altitudinal changes in life form and radial thickening growth of stems in *Rhododendron* plants. (a) Relationship between tree height and growing altitude. (b) Relationship between leaf length and tree height. (c) Relationship between annual radial growth of stems and growing altitude. (d) Difference in annual radial growth of stems between three different habitats and life forms. Data are fitted to power functions in (a), and linear functions in (b) and (c). Boxes in (d) represent the 50th percentile of the data set, and the lines represent the median. Bars of both sides of the box represent  $1.5 \times$  inter quarter range. Letters beside boxes in (d) represent significant difference at  $P < 0.05$  by ANOVA and Tukey's multiple comparison test.

### 3. Stem hydraulic properties.

#### 3-1. Efficiency of water transport in stems

A stem functions as the long distance water transport pathway from the roots to the leaves. The hydraulic performance of the stem depends strongly on the anatomical features of xylem (Tyree and Zimmermann 2002), with vessel diameter and the number of vessels (i.e., vessel density) particularly important for xylem water transport. The stem xylem of rhododendrons is diffuse porous, i.e. contains

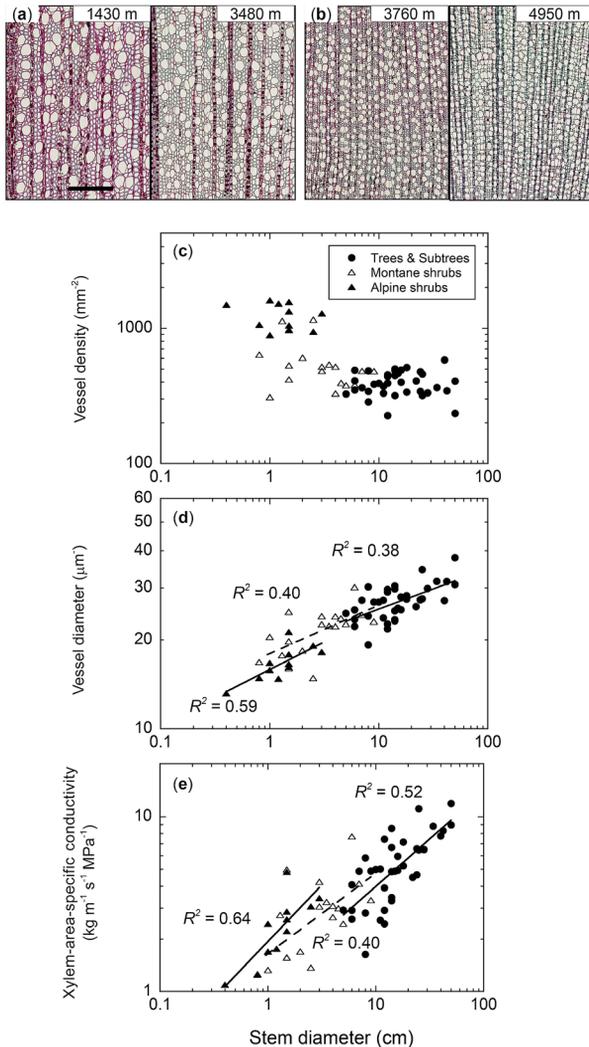
evenly distributed vessels of similar size throughout an annual ring (Figs. 3a, b). Across the 26 species, Noshiro *et al.* (1995) found a tight correlation of vessel diameter and density with stem diameter, but not with growing altitude. This inter-specific relationship suggests that ontogenetic constraints are more important for xylem vessel development than are the influences of the environment at different altitudes.

The present review re-analyzed this relationship separately for the three life forms (trees, montane shrubs and alpine shrubs). Vessel diameter was correlated positively with stem diameter for all three life forms (Fig. 3c), and there were no significant differences among the three relationships. By contrast, no significant correlations of vessel density with stem diameter were detected, for any of the three groups (Fig. 3d). Notably, vessel density of alpine *Rhododendron* shrubs was apparently larger than for the other two life forms (mean  $\pm$  1 s.e. of vessel density:  $397 \pm 12.9$ ,  $593 \pm 58.1$  and  $1240 \pm 77.7$  for trees, montane shrubs and alpine shrubs, respectively). These results suggest that the xylem vessel density of alpine shrubs was determined in an ontogenetic manner differently from the other two groups, while vessel diameter was produced by a common ontogenetic constraint.

According to the laws of hydrodynamics, water conductivity on a xylem area basis ( $k_x$ ) is calculated using vessel diameter and density (Tyree and Zimmermann 2002), normalized by stem segment length and conductive xylem area, allowing inter-specific comparison. In all three groups  $k_x$  was correlated with stem diameter, but the relationship of alpine rhododendrons differed significantly from those of rhododendrons growing at lower altitude (Fig. 3e). The regression line of alpine species was located above those of the other two groups, indicating that an alpine stem has a higher water transport capacity for a given stem diameter. At high-altitude habitats, freeze-thaw induced cavitation likely occurs through late autumn to winter and to early spring seasons (see next paragraphs for details), and so it seems reasonable that the higher stem water transport capacity of alpine rhododendrons is achieved by a higher vessel density in a stem, rather than wider vessels, which are susceptible to freeze-thaw induced cavitation.

### 3-2. *Stability of water transport in stems*

Stability of stem water transport is associated with higher resistance to xylem cavitation. Two abiotic stresses induce cavitation, or the entry of air bubbles into functional vessels. One is drought stress, and another is freeze-thaw cycles. Strong xylem tension in a drought-stressed plant can pull air into functional, water-filled conduits from adjacent air-filled conduits, through nanoscale pores on pit membranes (air-seeding, Choat *et al.* 2008). The threshold pressure of air entry is inversely proportional to the pore dimension in pit membranes (Tyree and

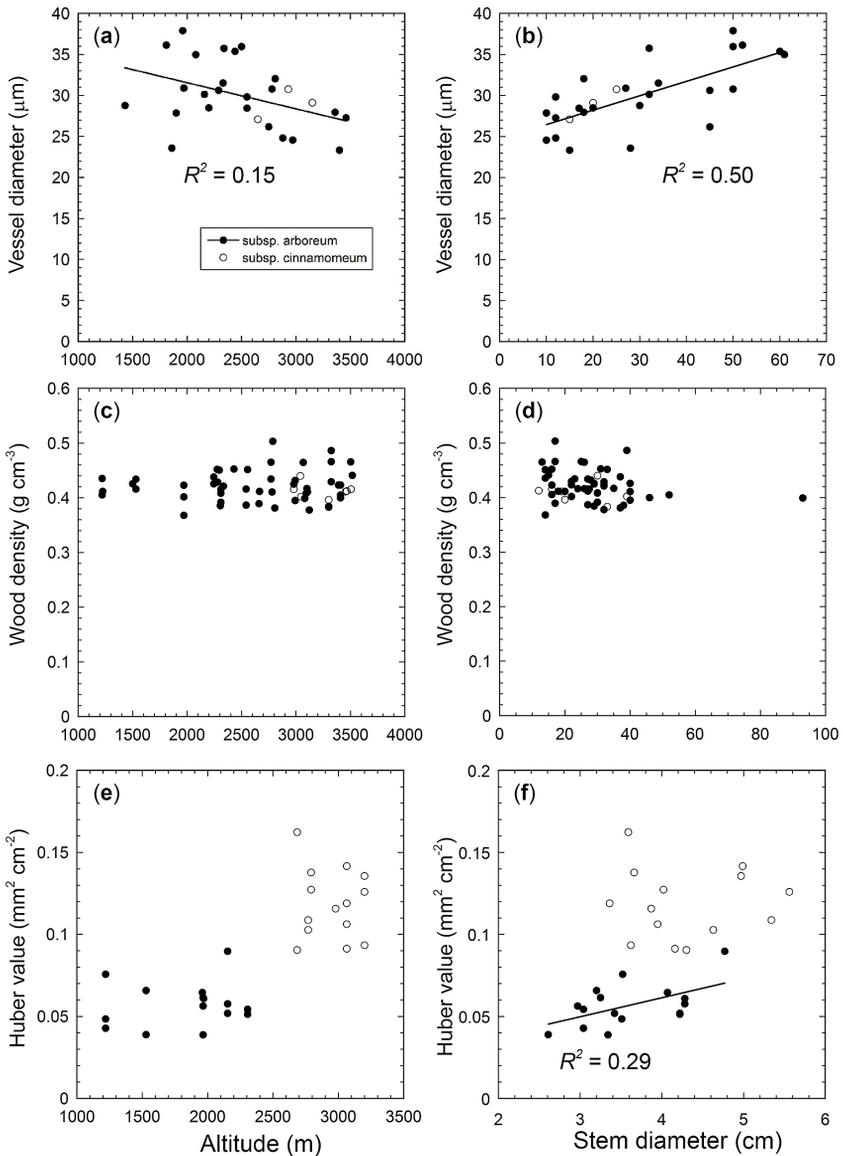


**Fig. 3.** Inter-specific variations in xylem anatomy and water conductive capacity of stem xylem (hydraulic conductivity) in rhododendrons. (a) Cross sections of *R. arboreum* stem xylem. (b) Cross sections of *R. anthopogon* stem xylem. (c) Relationship between vessel density and stem diameter among three life forms of rhododendrons. (d) Relationship between vessel diameter and stem diameter among three life forms of rhododendrons. (e) Relationship between xylem-area-specific conductivity and stem diameter among three life forms of rhododendrons. Closed circles solid line, and open and closed triangles in (c) - (e) represent tree species, montane shrub species, and alpine shrubs, respectively. Data in (d) and (e) are fitted to power functions. The fitted relationships in (d) are not different among the three life forms by ANCOVA test at  $P < 0.05$ . The fitted relationships of the alpine shrubs in (e) are different significant from the tree and subtree species by ANCOVA test at  $P < 0.05$ . Scale bar in (a) is 200  $\mu\text{m}$  and applies also to the three other images.

Zimmerman 2002), because the meniscus between water in a functional vessel and an air-filled vessel is retained by the surface tension of water. In addition to drought stress, freeze-thaw cycles of stem xylem sap also induce xylem cavitation (Sevanto *et al.* 2012, Sperry *et al.* 1994). In freezing of the stem xylem, gas bubbles appear in xylem sap due to their lower solubility at lower temperatures. After the thawing of stem xylem, gas bubbles expand under strong xylem tension, creating a large air volume that can fill a vessel lumen. Resistance to xylem cavitation can be determined by periodic measurements of stem conductivity and xylem tension in a drying stem. Resistant stem xylem retains its conductivity under strong xylem tension. The point of 50% loss of stem hydraulic conductivity ( $P_{50}$ ) is a common index of resistance to xylem cavitation. Previous studies have indicated that large conduit diameter is negatively related to resistance to xylem cavitation, whether induced by drought stress or freeze-thaw cycles (Christman *et al.* 2012; Hacke *et al.* 2006; Sperry *et al.* 1994; Taneda and Tateno 2005). The other factor, wood xylem density, is empirically positively related to resistance to drought-induced cavitation across many plant species (Hacke *et al.* 2001).

In rhododendrons, resistance to xylem cavitation has been measured in *R. macrophyllum* (Cordero and Nilsen 2002, Lipp and Nilsen 1997), *R. maximum* and *R. catawbiense* (Cordero and Nilsen 2002), and *R. ferrugineum*, *R. hirsutum*, and their hybrid (*R. ×intermedium*) (Mayr *et al.* 2010). The  $P_{50}$  values ranged between -1.75 MPa and -3.24 MPa, which would be considered moderate resistance compared to other angiosperms. These values were associated with drought conditions at the growing sites, but the correlation with growing altitude has not been reported. Lipp and Nilsen (1997) and Cordero and Nilsen (2002) have reported xylem dysfunction in winter, and severe inhibition in water transport was found in *R. catawbiense*, and in sunny plants of *R. macrophyllum* having vessels with wider diameters.

Because  $P_{50}$  data are not available for Nepalese rhododendrons, hydraulic stability was evaluated using their morphological traits. Vessel diameter was small in rhododendrons growing at high altitude, both for tree and shrub species (Fig. 2a). Thus, alpine *Rhododendron* shrubs should be more resistant to xylem cavitation induced by both drought stress and freeze-thaw cycles, at least in a basal part of stems. Intra-specific trends in the stability of xylem water transport were assessed in *R. arboreum* growing in eastern Nepal, classified into three subspecies, subsp. *arboreum*, subsp. *cinnamomeum*, and subsp. *roseum*. Vessel diameter of *R. arboreum* subsp. *arboreum* and subsp. *cinnamomeum* calculated from data of Noshiro and Suzuki (1995) decreased with increasing growing altitude, as in inter-specific variation (Figs. 4a). However, wood density measured from wood



**Fig. 4.** Altitudinal changes in stem morphological traits related to resistance to xylem cavitation among two subspecies of *R. arboreum*. (a) Relationship between mean vessel diameter and growing altitude. (b) Relationship between mean vessel diameter and stem diameter. (c) Relationship between wood density and the growing altitude. (d) Relationship between wood density and stem diameter. (e) Relationship between Huber value and growing altitude. (f) Relationship between Huber value and stem diameter. Closed and open circles in (a) – (f) represent subsp. *arboreum* and subsp. *cinnamomeum*, respectively. Data in (a), (b) and (f) are fitted to linear regressions.

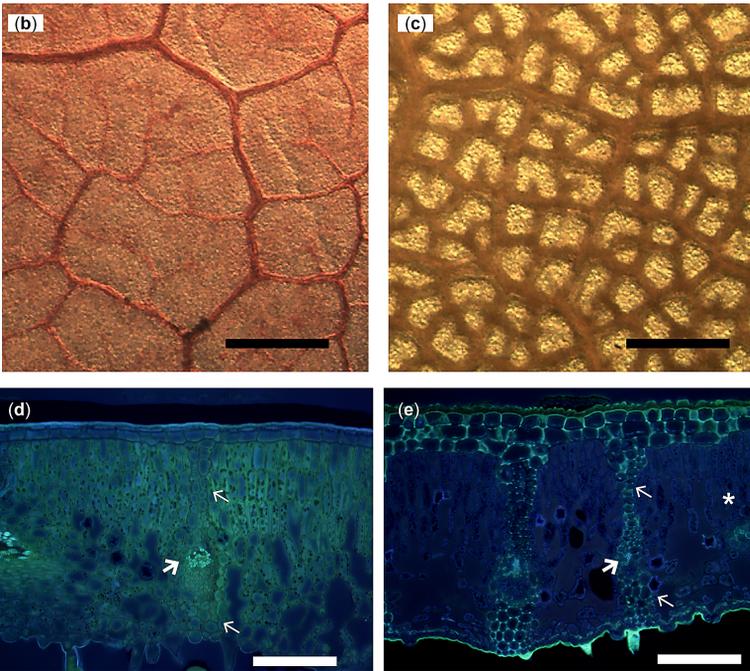
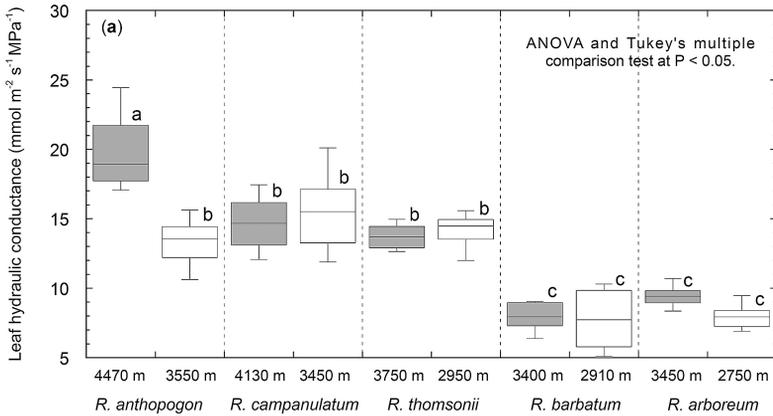
cores collected by a field trip to Rolwaling in 2007 was correlated neither with altitude nor stem diameter (Figs. 4c, d).

In addition, the Huber value was measured using specimens in the museum herbarium of the University of Tokyo, collected by the field trip to Rolwaling in 2007. The Huber value is an index of the water balance of a shoot, and is a ratio of the stem cross-sectional area at the current year shoot (water supply capacity) and the sum of the distal leaf area (potential water loss). The leaf area was calculated as a product of the length and width of the leaf lamina. The Huber value of a current-year shoot was seemingly correlated positively with altitude (Fig. 4e), but was significantly higher in subsp. *cinnamomeum* growing at higher altitudes than in subsp. *arboreum* growing at lower altitudes. Within subspecies, few correlations of the Huber value with growing altitude or stem diameter were found, except for the correlation between Huber value and stem diameter in subsp. *arboreum* (Figs. 4e, f). A high Huber value was linked to a smaller leaf area within a shoot, resulting in lower carbon gain. However, a high Huber value ensures higher stability of water balance in the shoot, because of a higher water-conducting area of xylem relative to the transpiring area of leaves. Positive correlations of Huber value with altitude have been reported in other woody species (Hernandez-Calderon *et al.* 2014). Furthermore, shrubs with small leaves show high Huber values (Tyree and Zimmermann 2002). Thus, the altitudinal trends of Huber value observed in *R. arboreum* may be applicable to inter-specific variations of rhododendrons. As with inter-specific variations, intra-specific trends of *R. arboreum* showed that the morphological traits of xylem and shoots reflect the conservative water use in *Rhododendron* species found at higher altitude.

## 4. Leaf hydraulic properties

### 4-1. Efficiency of water transport in leaves

Within a leaf, water flows first through xylem vessels in a petiole and veins and then from the xylem, through parenchyma tissue, to evaporation sites in mesophyll tissue. Leaf hydraulic resistance, which is inverse to hydraulic conductance ( $K_{\text{leaf}}$ ), accounts for 30% of the resistance in the whole plant pathway, suggesting a great impact on plant water use (Sack and Holbrook 2006). Among five rhododendrons occurring at different altitudes (*R. arboreum*: 2750–3450 m; *R. barbatum*: 2900–3400 m; *R. thomsonii*: 2950–3750 m; *R. campanulatum*: 3450–4130 m; and *R. anthopogon*: 3550–4470 m), a significant correlation was found between  $K_{\text{leaf}}$  and the growing altitude (Taneda *et al.* 2016), but within a species,  $K_{\text{leaf}}$  of plants from higher altitudes was not different from that from lower altitudes, except with *R. anthopogon* (Fig. 5a)



**Fig. 5.** Altitudinal changes in water conductive capacity of leaf (leaf hydraulic conductance) and leaf morphological traits. (a) Box-plot of leaf hydraulic conductance among five *Rhododendron* species from the lowest to the highest altitudinal habitats. Letters beside boxes show significant difference by Tukey's multiple comparison test at  $P < 0.05$ . (b) and (c) Cleared leaf lamina of *R. anthopogon* and *R. arboreum*, respectively. (d) and (e) Thin cross-section of *R. anthopogon* and *R. arboreum* leaves observed with a florescent microscope, respectively. Pale blue florescence comes from lignified cell walls. Thick and thin arrows in (d) and (e) are vein xylem and the bundle sheath extensions, respectively. Asterisk in (e) represents the highest order vein without bundle sheath extensions. Scale bars in (b) and (c) are  $250 \mu\text{m}$ , and those in (d) and (e) are  $100 \mu\text{m}$  and  $200 \mu\text{m}$ , respectively.

Previous studies reported that vein length per area (VLA) is a key trait to explain inter-specific variation in  $K_{\text{leaf}}$  (Brodrribb et al. 200; Sack et al. 2013).  $K_{\text{leaf}}$  is associated positively with VLA, with higher VLA resulting in a shorter water transport distance through parenchyma tissue with high hydraulic resistance (Brodrribb et al. 2007). On the other hand, no significant relationship between  $K_{\text{leaf}}$  and VLA was found among the five *Rhododendron* species. Notably, *R. arboreum*, with the highest VLA, showed the lowest  $K_{\text{leaf}}$ , and *R. anthopogon* with the lowest VLA had the highest  $K_{\text{leaf}}$  (Figs. 5bc). Lignification on the bundle sheath cells was the most plausible candidate to constrain the relationship between  $K_{\text{leaf}}$  and VLA among the five *Rhododendron* species (Taneda et al. 2016). In *R. arboreum* leaves, bundle sheath extensions developed at all vein orders, except for vein endings, and the cells of bundle sheath and bundle sheath extensions were lignified in the primary walls (Fig. 5d). Unlike the Casparian band in the endodermis of roots (Enstone et al. 2003, North and Peterson 2005), in the bundle sheath of the veins the primary walls were lignified on all aspects of the cell. Contrastingly, the bundle sheath cells of *R. anthopogon* were not lignified even in the second-order veins, although the bundle sheath extensions developed up to higher-order veins (Fig. 5e). Lignin is a phenolic polymer with a hydrophobic property, and deposits of it fill the space between microfibrils in cell walls (Barros et al. 2015), thus inhibiting water transport. Consequently, although *R. arboreum* showed a high measured VLA, veins irrigating mesophyll cells may be highly limited in their water transport capacity because of the lignification of bundle sheath cells, resulting in a low  $K_{\text{leaf}}$  (Ohtsuka et al. 2018, Taneda et al. 2016). *R. barbatum* leaves with a low  $K_{\text{leaf}}$  showed the same lignification pattern as *R. arboreum* leaves. In the minor veins of *R. campanulatum* and *R. thomsonii*, lignification was found only in bundle sheath extensions on the abaxial side (Taneda et al. 2016), which are considered as a bypass between xylem veins and the epidermis, where stomata are located. These two species had middle values of  $K_{\text{leaf}}$  between *R. arboreum* and *R. anthopogon* from high altitude. The lignification on bundle sheath cells was great negative impact on leaf hydraulics of Nepalese *Rhododendron* species, but the merit of it is still unclear. One is to prevent leaking from the lower-order veins (upstream of leaf water transport), likely resulting in equal supply of water to any parts of a leaf. Another may be the mechanical reinforcement of bundle sheath cells. Lignified cells are solid against external force because of little leaking of water from cells under pressure (Niklas 1992). The positive correlation of  $K_{\text{leaf}}$  with altitude is associated with efficient carbon gain at the growing altitude, because a systematic increase in both  $K_{\text{leaf}}$ , leaf nitrogen concentration and stomatal pore index (stomatal width<sup>2</sup> × stomatal density, Sack et al. 2003) with the growing altitude were detected (Taneda et al. 2016). Leaf nitrogen concentration is related tightly with the content of Rubisco,

which is an enzyme for CO<sub>2</sub> assimilation (Makino 2003). High leaf nitrogen concentration is linked to a high maximum photosynthetic rate (Evans 1989). Through high stomatal conductance (as estimated by high stomatal pore index) a great amount of CO<sub>2</sub> can be obtained from the atmosphere, supported by high nitrogen availability. High stomatal conductance simultaneously induces a high rate of transpiration from a leaf. Dwarf alpine shrubs growing at a high density and with high transpiration rates can induce a high boundary layer resistance [i.e., a plain canopy of prostrate shrubs (see Fig. 1c) causes less mixing of air space just above the canopy, which impedes further movement of gaseous materials and heat from leaf surfaces]. When leaves received strong irradiance from sun at low latitudes, leaf temperature can rise sharply even in high altitude habitats, resulting in great transpiration demand (Körner 2003, Larcher *et al.* 2010). The substantial loss of water can be compensated for by a high capacity of leaf water transport. The leaves of alpine shrubs are adapted to the harsh alpine environment with an extremely short growing season by being able to continue photosynthesis without temporal stomatal closure at the midday.

#### 4-2. *Stability of water transport in leaves*

A decrease in  $K_{\text{leaf}}$  with drought has been reported across a wide range of plant species during the last decade (reviewed in Scoffoni and Sack 2017).  $K_{\text{leaf}}$  decreases due to xylem cavitation in leaf vein xylem (Johnson *et al.* 2012, Brodribb *et al.* 2016), reversible collapse of vessels in minor veins (Zhang *et al.* 2016), and shrinkage of the parenchyma cells in the bundle sheath (Scoffoni *et al.* 2017a). To date, two morphological traits have been proposed to determine the risk of leaf vein xylem embolism: leaf area (Scoffoni *et al.* 2011) and the ratio of the thickness of vessel cell walls to the diameter of the vessel lumen (TD ratio, hereafter). High TD values are associated with high strength and resistance to vessel implosion (Blackman *et al.* 2010). A small area leaf with great density of major veins is less susceptible to a decrease in  $K_{\text{leaf}}$ . A leaf with a higher TD ratio has more negative  $P_{50}$  values of  $K_{\text{leaf}}$  (i.e., more resistant to xylem embolism). Unfortunately, there is little information about the  $P_{50}$  values of *Rhododendron* leaves (except for  $P_{50} = -1.95$  MPa for *R. macrophyllum*, Johnson *et al.* 2012). Thus, the stability of leaf water transport was estimated by comparing leaf area and TD ratio among five *Rhododendron* species (Table 1). Leaf area ranged from 3.55 cm<sup>2</sup> for *R. anthopogon* to 57.6 cm<sup>2</sup> for *R. barbatum*, suggesting that the alpine *R. anthopogon* had the highest stability of leaf water transport. The TD ratio varied between 0.0123 and 0.0235 (Figs. 5a, b). Difference in the TD ratio was well explained by the lumen diameter of vessels, rather than differences in cell wall thickness. As in the case of leaf area, TD ratio was the highest in the alpine *R. anthopogon* growing at 4470

Table 1. Leaf morphological traits related to xylem cavitation

Species	Altitude (m)	Leaf area (cm <sup>2</sup> )	Wall thickness to Span ratio of minor vein	Estimated $P_{50}$ (MPa)	Hydraulic vessel diameter of midrib xylem (mm)
<i>R. anthopogon</i>	4470	3.86 (0.089)	0.0235	-6.18	8.44 (0.30)
<i>R. anthopogon</i>	3550	3.55 (0.223)	0.0206	-4.76	7.20 (0.25)
<i>R. campanulatum</i>	4130	36.4 (1.34)	0.0131	-3.02	15.5 (0.64)
<i>R. campanulatum</i>	3450	30.4 (1.67)	0.0123	-2.84	15.0 (0.48)
<i>R. thomsonii</i>	3750	37.1 (1.50)	0.0135	-3.11	13.7 (0.13)
<i>R. thomsonii</i>	2950	29.8 (0.62)	0.0160	-3.70	13.4 (0.31)
<i>R. barbatum</i>	3400	43.3 (1.37)	0.0171	-3.94	14.4 (0.32)
<i>R. barbatum</i>	2900	57.6 (2.80)	0.0131	-3.02	14.9 (0.47)
<i>R. arboreum</i>	3450	41.0 (1.75)	0.0161	-3.72	12.4 (0.32)
<i>R. arboreum</i>	2750	34.0 (1.49)	0.0143	-3.30	10.4 (0.21)

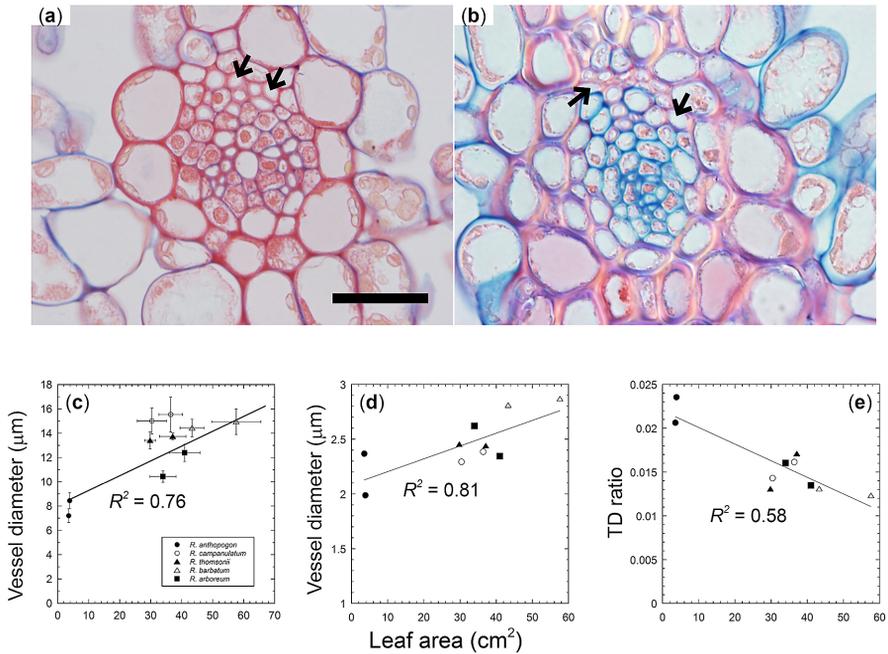
footnote:  $P_{50}$  represents leaf water potential causing 50% loss of leaf hydraulic conductance. Numbers in parentheses are 1 s.e.  $n = 4 - 6$ .

m. The  $P_{50}$  value estimated with a fitted equation by Blackman (2010;  $P_{50} = 230 \text{ TD} + 0.748$ ) was about -6 MPa, which is extremely high (Table 1). In addition, X-ray CT observations indicated that resistance to xylem cavitation is associated with the vessel diameter of the midrib vein (Scoffoni *et al.* 2017b). *R. anthopogon* growing at the highest altitudes should also have the highest stability of xylem transport, due to its narrowest diameter of the vessels (Table 1).

All the indices of hydraulic stability were highest in *R. anthopogon* growing at a highest altitude. The diameter of vessels in a leaf vein is ontogenetically dependent on the leaf area (Taneda and Terashima 2012). For the five *Rhododendron* species, variations in the vessel diameter of the midrib and of the minor veins are explained by leaf area (Figs. 6c, d). The high hydraulic stability of *R. anthopogon* might be produced by the ontogenetic constraint, i.e. small leaves attached on small plants had narrow vessels in its leaf venation.

## 5. Efficiency of water transport in the whole-plant pathway

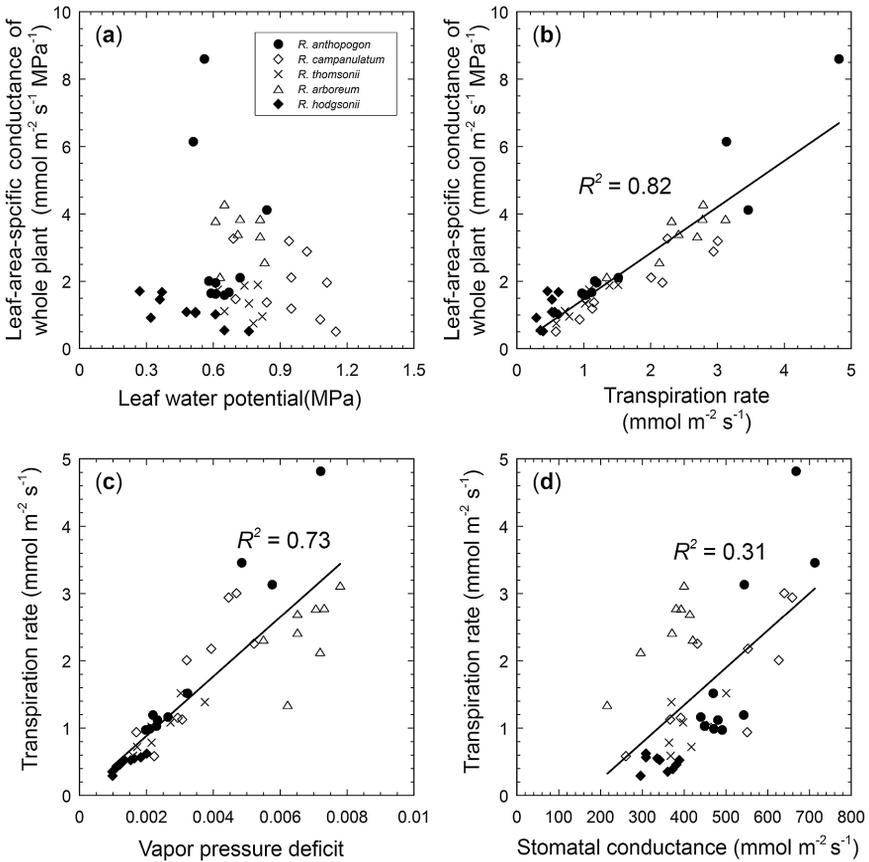
Finally, inter-specific differences in whole plant hydraulic conductance (i.e. transpiration rate on an area basis divided by the difference in water potential between soil and leaf,  $K_w$  hereafter) with altitude are examined. These parameters were measured in the morning. Notably, clear trends were not obtained because  $K_w$  varied four to eight fold for *R. campanulatum* and *R. anthopogon*, respectively. The difference in  $K_w$  was associated with a difference in transpiration rate, rather than with differences in leaf water potential (Figs.7a, b). The results of ANCOVA analyses indicated that  $K_w$  increases with increasing transpiration rate in the same proportions for all of these *Rhododendron* species, except for *R. campanulatum*.



**Fig. 6.** Leaf morphological traits related to resistance to hydraulic dysfunction under drought stress among five *Rhododendron* species. (a) and (b) Thin cross-sections of vascular bundles in highest-order veins of *R. anthopogon* and *R. arboreum*, respectively. Sections were stained with a mixture of safranin and astrablue solutions. (c) Relationship between the diameter of a midrib-xylem vessel and leaf area. (d) Relationship between the diameter of a highest-order-vein xylem vessel and leaf area. (e) Relationship between the third power of ratio of wall thickness to lumen diameter of a vessel (TD ratio) and leaf area. Data in (c)–(e) are fitted to linear regressions. Arrows in (a) and (b) indicate vessels. Scale bar in (a) is 20  $\mu\text{m}$  and applies to (b). Symbols in (c)–(e) represent different species shown in (c). Bars at each point in (c) show 1 s.e.

The difference in transpiration rate could be explained strongly by vapor pressure deficit (Figs. 7c, d). These results suggest that the increase in  $K_w$  allows a transpiring plant to continue photosynthesis, even under high transpiration demand, without a decrease in leaf water potential inducing stomatal closure or the occurrence of xylem cavitation (Mayr *et al.* 2010).

There are two plausible mechanisms for an increase in  $K_w$  with transpiration rate. Water stored in stems is available with a lower friction than is the water absorbed by roots from soil, although the amount of stored water is limited (Scholz *et al.* 2007). In the morning when the measurements were conducted, the stored water might be available for transpiration from leaves without a large decrease in leaf water potential occurring in response to an increase in



**Fig. 7.** Inter-specific variations in whole-plant water transport capacity (hydraulic conductance) among five *Rhododendron* species. (a) Relationship between whole-plant hydraulic conductance and leaf water potential. (b) Relationship between whole-plant hydraulic conductance and transpiration rate on an area basis. (c) Relationship between transpiration rate and vapor pressure deficit. (d) Relationship between transpiration rate and stomatal conductance. The pooled data of all species are fitted to linear functions. Different symbols represent different species as shown in (a).

transpiration rate. The other mechanism is a short-term response due to the regulation of activity of aquaporins. Water passes through the plasma membrane in the bundle sheath of a leaf (Shatil-Cohen *et al.* 2011), and in the endo- and exodermis in a root (North and Peterson 2005). These tissues have high friction against water transport, so these tissues also include cells with high expressions of aquaporins (Gambetta *et al.* 2013, Shatil-Cohen *et al.* 2011). [Editor’s note: Aquaporins are highly-specific membrane-bound channels that provide a low resistance pathway for water to move across cell membranes.] For roots of rice

and popular tree (*Populus trichocarpa* × *P. deltoides*), concomitant increases in root hydraulic conductance and gene expression of aquaporins are detected after the onset of a light period inducing a high rate of transpiration (Sakurai-Ishikawa *et al.* 2011, Laur and Hacke 2013). In addition, Simonin *et al.* (2015) implied the involvement of aquaporins for an increase in  $K_{\text{leaf}}$  with increasing transpiration rate. Unfortunately, no previous studies have been conducted on the function of aquaporins in rhododendrons. Further measurements are expected to prove the involvement of aquaporins on whole plant water transport as an adaptation to high-altitude habitats in rhododendrons.

## 6. Conclusions

The present review assessed altitudinal changes in the efficiency and stability of water transport in rhododendrons occurring in eastern Nepal, based on morphological traits of leaf and stem xylem. Due to the humid climate of eastern Nepal, transpiration demand potentially decreases with increasing altitude. A small transpiration demand theoretically allows the low water transport and low stability of plant water transport with a low carbon cost. However, both capacity and stability of water transport were greatest in alpine rhododendrons growing at high altitudes. High vessel density in these alpine plants resulted in a greater capacity of stem water transport than required by rhododendrons from lower altitude habitats. Leaf hydraulic conductance was also greatest in species found growing above the treeline. Xylem traits and shoot morphology suggest more conservative water use by rhododendrons growing at high altitudes. Furthermore, all morphological indices examined indicate alpine rhododendrons had the highest stability, being strongly resistant to a decrease in leaf hydraulic conductance with drying. The tight correlations of these morphological indexes with leaf area imply that ontogenetic constraints cause narrow vessels in small leaved alpine *Rhododendron* species, leading to their high resistance to xylem cavitation. The stability of plant water transport is threatened under severe alpine climate conditions, due to a sharp increase in transpiration demand owing to strong irradiance and high boundary layer resistance, and the freeze-thaw cycle of xylem sap and soil in the winter. Occurrence of xylem cavitation can be a fatal event for alpine plants, because an extremely slow radial growth of stems cannot compensate for the loss of hydraulic conductance by producing new vessels quickly. During a recovery, plants will suffer from water deficiency causing a decrease in carbon gain and in the worst, plant death. Therefore, these hydraulic properties are adaptive to survival in high-altitude habitats.

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