

**Vegetative Anatomy of *Rhododendron* with a Focus on a Comparison between Temperate
and Tropical Species**

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Abstract

Rhododendron is a monophyletic group that inhabits many different climates. One clearly defined diversification was from temperate ancestors into tropical habitats. The focus of this work was to explore leaf and stem anatomical traits in relation to habitat (temperate and tropical) and elevation of the native range. A closely-related group of *Rhododendron* was selected to reduce variation in genetic history and reveal environment-associated adaptive traits.

Vessel anatomical traits of *Rhododendron* accessions were assayed for the trade of between safety (protection against catastrophic failure) and efficiency (high theoretical conductivity). *Rhododendron* wood and vessels were found to be relatively safe. The metrics of wood efficiency were higher for the tropical species. Thus, a trade-off between safety and efficiency was found although the wood of *Rhododendron* is characterized as highly safe.

Leaf anatomical traits of *Rhododendron* were assayed for habitat and elevation. Leaves on tropical species were thicker and denser compared with temperate species. Idioblasts were always found in tropical leaves but not in temperate species. Leaves of tropical species were more xeromorphic (drought tolerant) than those of temperate species. Increasing elevation of the native range did not influence leaf anatomical traits.

Idioblast abundance and leaf water relations traits were assayed for tropical *Rhododendron* species. Idioblast expression varied from 5% to 28% and stomatal pore index varied from 0.08 to 3.3. Idioblast expression was highly correlated with leaf succulence, and water deficit at the turgor loss point. Idioblast expression was positively associated with leaf capacitance for thin (< 0.5 mm) leaves. Thus, idioblasts can serve as a water buffer for relatively thin leaves.

Synthesis –Wood traits of evergreen *Rhododendron* shrubs reflect adaptation for safety. Although the tropical species have significantly higher efficiency, wood safety is still the dominant feature. The implication of high wood safety is constrained water flow and a potential for low water potential. Both leaf succulence and the presence of idioblasts in thin leaves enhances leaf capacitance and provides some buffering against short-term drought. These leaf adaptations in tropical *Rhododendron* shrubs likely reflect the abundance of epiphytes in this group.

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General Audience Abstract

Rhododendron is a very diverse genus that is found in many different habitats from arctic to tropical. However, most of the species are evergreen with a slow growth rate. The goal of this study was to explore the variation in wood and leaf anatomical traits in order to explain how these plants can succeed in so many different habitats. The vessels in wood of temperate species were found to be very small. Although the size of the vessels increased for tropical species, they were still small relative to many other species. Surprisingly, leaf traits suggested greater drought tolerance for tropical species compared with temperate species. A unique anatomical trait called idioblasts was found only in leaves of tropical species. Idioblasts were very large cells, found just below the upper epidermis, which occupied up to 30% of the leaf volume. Idioblasts were found to help buffer water loss for thin tropical leaves. In summary, *Rhododendron* wood constrains water flow for plants in all habitats, which will induce water stress in warm or dry areas. Consequently, leaves have drought tolerance traits in tropical regions. Therefore, anatomical traits of wood and leaf help explain how *Rhododendron* species can occupy a wide diversity of habitats.

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ATTRIBUTIONS

Chapter 1 –Vegetative functional traits, *Rhododendron* taxonomy and the research plan:

To present background information needed to understand functional traits of leaves and stems with a focus on evergreen shrubs and the taxonomic relationships in

Rhododendron. 1) Erik T Nilsen, PhD is currently professor of Plant Ecology in the Department of Biological Sciences at Virginia Tech. Dr. Nilsen provided mentorship and literature concerning the genus *Rhododendron* and functional trait theory.

Chapter 2 - Exploration of wood anatomical traits among accessions of *Rhododendron*: A focus on the relationship with the elevation and climate (temperate vs. tropical) of the

native range. 1) Erik T Nilsen, PhD is currently professor of Plant Ecology in the Department of Biological Sciences at Virginia Tech. Dr. Nilsen assisted with experimental design, helped attain accession material and assisted in data analyses. 2) Dr. Ann Norris, and Kyle Mirabile, assisted with stem sectioning, permanent slide preparing, and Nikon NIS-Elements Basic Research program.

Chapter 3 – Exploring leaf anatomical traits of *Rhododendron* accessions: A focus on the relationship with the elevation and habitat (temperate vs. tropical) of the native range. 1)

Erik T Nilsen, PhD is currently professor of Plant Ecology in the Department of Biological Sciences at Virginia Tech. Dr. Nilsen assisted with experimental design, helped attain accession material and participated in some sample preparation and analysis. 2) Melanie Taylor was a MAOP student who helped with sample preparation and image capture. 3) Courtney Johnson was an undergraduate Biochemistry major who helped preparing slides.

Chapter 4 - The significance of idioblast to bulk leaf water relations in tropical accessions of *Rhododendron*. 1) Erik T Nilsen, PhD is currently professor of Plant Ecology in the Department of Biological Sciences at Virginia Tech. Dr. Nilsen assisted with experimental design, helped attain accession material performed the pressure volume curves, and assisted in data analyses. 2) Melanie Taylor was a MAOP student who helped with image capture and data base formation. 3) Courtney Johnson was an undergraduate Biochemistry major who helped preparing slides.

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CHAPTER 1

Vegetative functional traits, *Rhododendron* taxonomy and the research plan

1.1 Importance of leaf and stem anatomical traits to plant fitness

Vegetative functional traits are characteristics of plants that strongly influence or regulate important functions related to plant fitness. Arguably, one of the most important functions of plants is photosynthesis. Most plants acquire carbon resources from photosynthesis, which in most cases, happens in leaves. Even though photosynthesis is based on biochemical process, leaves are the physical environments where photosynthesis occurs. There are many anatomical traits in leaves or other associated factors such as water balance that influence photosynthesis. Leaf stomata are one example of a functional anatomical trait, because they control carbon dioxide exchange, regulate transpiration and influence the water potential gradient. Leaf chemical characteristics can protect leaves against herbivores that could remove or destroy photosynthetic potential. The protective chemicals can be accumulated inside the leaf or outside as in glandular trichomes. Many of these anatomical, morphological or chemical traits of leaves constitute the phenotypic variation that promotes photosynthesis and plant fitness in different climatic conditions.

The Leaf Economics Spectrum (LES) hypothesis was clearly described (Reich 2014) as a unifying theory of the relationship between leaf traits and climate. This theory is based on a trade-off between leaf production investment and plant fitness (Figure 1.1) Faster growing plants have higher nitrogen content in leaves with a lower investment in structure and do not last as long, but yield higher photosynthesis rates. Leaves on slow-

growing plants tend to last longer, have higher structure (higher mass per area), a lower nitrogen content, and a lower photosynthetic rate. However, these relationships are based on log-log plots, which hide the large variation in these correlations within individual taxonomic groups, climates or habitats. The large amount of hidden variation suggests that there are other leaf functional traits than those included in the LES that may determine the variation seen in the LES theory. For example, slow growing evergreen shrubs reside in a diversity of different habitat types from tropical lowland to arctic shrub land. Variation in leaf anatomy and morphology among evergreen plants that reside in these different habitat types may be more important to their respective fitness than simply the slow-growth syndrome traits defined by the LES.

Xylem traits also are important for determining the fitness of plants in different climates. A majority of a plant stem is composed of xylem, which is the water transporting tissue. One of the most important functions of a stem is to transport water upward and thus supply water to foliage. Vessel elements, which are subunits of xylem conduits need to maintain hydraulic conductivity from root to shoot. Cavitation in vessel elements, caused by embolisms inhibits hydraulic conductance. The main reasons for embolism are drought and freeze-thaw cycles. Larger diameter vessels ($> 30 \mu\text{m}$) are more susceptible to embolism compared to smaller vessels. In addition, the greater the number of vessel wall pits the greater the vulnerability to drought-induced embolism. Plants in temperate habitats have to cope with frequent freeze-thaw cycles that can induce embolism during the winter. Species with smaller vessel diameter are considered “safer” because such plants are less susceptible to embolism (Sperry and Sullivan 1992), particularly in response to freeze-thaw cycles.

In contrast, the potential magnitude of hydraulic capacitance (termed efficiency) is dependent upon vessel density and vessel diameter. Plants that have larger vessels and a greater density of functional vessels have greater efficiency. Thus, there is a tradeoff between “safety”, smaller vessel diameter, and “efficiency”, larger vessel diameter. Hydraulic safety may be more important to temperate species, while hydraulic efficiency may be more important to tropical species. Therefore, this trade-off between stem efficiency and safety may be an important way that evergreen shrubs (for example *Rhododendron* species) adapt to major climatic differences such as the difference between temperate and tropical climates.

1.2 Problem of determining the functional significance of anatomical traits

It is clear that anatomical traits of leaves and stems can be important ways that plants adapt to their environment. Although this concept has been studied many times, there is often a complicating factor in these studies. The ultimate cause of variation in anatomical traits is both the environment and the genome. Environment can induce some anatomical variation, yet studies in common gardens often point to significant genetic regulation of anatomical traits. Variation in anatomical traits can often evolve through microevolution where natural selection is acting upon genetic material. Therefore, the phylogenetic history of a lineage can be an important cofactor that influences the variation in traits between environments. This confounding effect of phylogenetic history is a significant problem when trying to understand if the variation in an anatomical trait has adaptive significance.

Three of the ways of addressing the problem of separating phylogenetic history from environment-induced microevolution of anatomical traits are:

1) Study anatomical traits of a monophyletic group that has members in each of the selected environments. If traits are significantly different between the environments, then these are functionally significant for each environment because the confounding effect of phylogenetic history is minimized.

2) Study anatomical traits of several different phylogenetic groups in each of the different environments. If anatomical traits are consistent among different groups within each environment and significantly different between environments, then the anatomical differences most likely have functional significance due to convergent evolution among groups that have independent phylogenetic history.

3) Map anatomical traits on a known phylogenetic diversification from one environment into another environment. If the anatomical traits change quantitatively along the diversification axis, then the traits most likely have functional significance.

The goal of this study was to examine how anatomical traits of leaves and stems vary among members of a monophyletic group (*Rhododendron*) that has both temperate and tropical species. Thus, technique one was used to minimize the confounding effect of phylogenetic history on the comparison of anatomical traits for species in temperate and tropical habitats.

1.3 *Rhododendron* classification

There are approximately 1,000 species recognized in *Rhododendron* (Ericaceae), which encompass a wide diversity of growth forms and growth habitats (Davidian 1995, Chamberlain and Royal Botanic Garden 1996, Cox and Cox 1997). Growth forms include ground vines, bog perennials, small epiphytic shrubs, large shrubs, small trees, and large canopy trees. The native ranges of *Rhododendron* species occur from sea level to 5,000 m in elevation (Argent 2006). *Rhododendron* species are distributed in tropical, temperate, and arctic habitats and occur in Europe, North America, the Himalayan region and throughout Asia. The restriction of most *Rhododendron* species to the northern hemisphere suggests a Laurasian origin. In fact, there are only two *Rhododendron* species in Gondwanan regions (Australia), and those are relatively new colonists.

Efforts to classify species of *Rhododendron* extends back to Linnaeus who named the first *Rhododendron* species including *Rhododendron maximum* L. (Blacksburg, VA's local dominant species). The first thorough treatment of the genus was prepared by Sleumer (1949), based primarily on herbarium specimens and a few excursions to Indonesia and Malaysia. Sleumer's taxonomy of *Rhododendron* expanded on the treatment by Linnaeus. These early efforts to classify *Rhododendron* species were focused on accounts by explorers, living collections and herbaria specimens in Europe (Sweden, Netherlands, and British Isles) and culminated in a most thorough treatment from the Royal Botanic Garden Edinburgh, prepared by Chamberlain (Chamberlain and Royal Botanic Garden 1996).

The morphological classification of *Rhododendron* used by Chamberlain and Royal Botanic Garden (1996) is based on leaf scale morphology, leaf longevity (deciduous or evergreen), floral morphology and branching architecture. The presence or absence of leaf scales forms a major dichotomy in *Rhododendron* resulting in lepidote species (with leaf scales) referred to as subgenus *Rhododendron* and elepidote species (without leaf scales) were placed in several subgenera including *Hymenanthes*. Subgenus *Rhododendron*, which includes almost half of all species in the genus *Rhododendron*, includes the majority of tropical species. Subgenus *Hymenanthes* contains a majority of all elepidote species. The other 6 subgenera of elepidotes have much fewer species than *Hymenanthes* (Table 1.1). Chamberlain grouped *Rhododendron* into 8 subgenera and 12 sections. Of the 8 subgenera, *Rhododendron* and *Hymenanthes* were considered the most monophyletic and they contained the majority of species in the genus (Table 1.1). Subgenus *Rhododendron* was divided into 3 sections (*Rhododendron*, *Pogonanthum*, and *Vireya*) while subgenus *Hymenanthes* had only 1 section (*Ponticum*). Subgenus *Vireya* was dominated by tropical species while *Pogonanthum* and *Rhododendron* were dominated by temperate species. A majority of species in *Hymenanthes* had temperate range and were distributed among the various continental locations associated with a Laurasian origin (e.g. Europe, North America, Japan, Taiwan, China, Russia). (Table 1.1)

Over the past 15 years there have been multiple attempts to use molecular techniques to classify *Rhododendron* species and define phylogenetic patterns. *Rhododendron* has been shown to be a strongly monophyletic group in the Ericaceae and should include both *Ledum* and *Menziesia* (Kron and Judd 1990a). Moreover, subgenus *Therorhodon* was a sister group to all other *Rhododendron* groups (Kron 1997,

Kurashige et al. 2001), based on the ITS1 and 2 spacer regions. Later, subgenus *Rhododendron* was determined to be polyphyletic and that *Pentanthera* (deciduous shrubs) were aligned inside the *Rhododendron* polyphyletic group (Kron and Johnson 1998). These data were considered preliminary because only a few taxa were used for each group. A most parsimonious tree for the genus *Rhododendron* (Goetsch et al. 2005) was based on consensus sequences of the *RPB2-1* nuclear gene from 87 species. The result of this analysis reduced the number of subgenera to five, but retained subgenus *Rhododendron* as a monophyletic group with three sections including *Vireya*. (Table 1.2)

Throughout the history of morphological and molecular studies of *Rhododendron* classification, *Vireya* has been considered a strong monophyletic group aligned within subgenus *Rhododendron*. Chamberlain's morphological taxonomy was updated by Argent in relation to section *Vireya* (Argent 2006). Argent divided *Vireya* into 7 subsections based mostly on floral and bud traits (Table 1.3). Also, Brown et al. (2006) published a molecular treatment of *Vireya* based on ITS sequences that showed many of the subsections identified by Argent were polyphyletic. In fact, the phylogenetic relationships seemed to be more governed by geographic range than morphological traits. In 2011 a new molecular treatment of *Vireya* was published (Goetsch et al. 2011) that supported 4 monophyletic subsections in *Vireya* (Figure 1.2) in ancestral to derived order (*Discovireya*, *Pseudovireya*, *Malayovireya*, *Euvireya*). Craven et al. (2011) suggested that the correct placement of *Vireya* was at the sectional level under the name *Schistanthe* because of historical naming protocols (Table 1.3).

Argent (2015) explains the controversy about the placement of *Vireya* in *Rhododendron*. His placement of this group at the subgenus level follows the original

work by Sleumer in 1949. This placement is based on enhancing the ability to name species and place them into groups based on strong morphological, and chemical grounds. Therefore, Argent's 2015 treatment offers a practical way to divide the large group into subunits so that species can be uniquely identified and named. In contrast, other efforts exclusively used DNA sequence similarities to identify monophyletic arrangements of entities in this group (Brown et al. 2006, Craven et al. 2011, Goetsch et al. 2011). The weak congruence between the morphology and molecular classifications may be due to the fairly recent diversification of this group into tropical southeast Asia. In fact, species isolation may be incomplete because Argent (personal communication) speaks of many hybrid swarms in natural habitats, and entities identified as species easily hybridize in culture. The group will be defined as subgenus *Vireya* throughout this report based on the most recent treatment by Argent (2015). However, when consider how traits of plants changed during their diversification into the tropical region, the analysis is based on the molecular evidence (ITS sequence) for that diversification event (Goetsch et al. 2011). (Table 1.3)

1.31 Origin of *Rhododendron* and its initial range—Fossils of *Rhododendron* taxa pollen were first reported in 1981 (Muller 1981) in the upper Paleocene, which dates to about 60 million years ago (Zetter and Hesse 1996). *Rhododendron* pollen is easily identified because it is found in groups of four (tetrads) simplifying the ability to identify them. Also, the fossil record indicates that *Rhododendron* species existed 50 million years ago in Alaska. Unfortunately, *Rhododendron* fossils are not abundant, in the record which may be due to their upland range and well-drained habitat (Irving 1993).

The climate was warmer and more stable during early *Rhododendron* history in the Laurasian circumboreal forest (Irving 1993). Plants could flourish at relatively high latitude and elevation compared to the present due to the warmer climate. During this time, *Rhododendron* species were uniformly distributed across Laurasia compared to the recent period. Global climate change, continental drift and the rise of mountains are considered three major factors that have influenced the diversification of *Rhododendron*. These three processes fractured the geographic uniformity of optimal growing conditions for *Rhododendron* species (Irving 1993). For example, during continental movement upland terrains emerged that were hostile habitats for *Rhododendron* genotypes. About 20 million years ago, North America and Europe were moving apart creating the Atlantic Ocean and isolating groups of Laurasian species including *Rhododendron*. Also, the Himalayas were forming by the movement of the Indian plate into the Asian plate. The mountain relief and high-island archipelago formed by these continental movements created new habitat conditions and isolated many relict groups of Laurasian species (Milne 2004). In general, continental movement and global climate change created many new environments and new topography that isolated many groups of *Rhododendron* species resulting in some diversification events and some relict species (Irving 1993).

1.32 Diversifications of *Rhododendron* species—At its origin, *Rhododendron* ranged throughout moist, warm-temperate areas of the northern hemisphere because of its original Laurasian heritage in the circumboreal forest. The current diversity and global distribution pattern of *Rhododendron* species reflects both its origin, species diversification into new habitats and isolations resulting in relict distributions. Within the

current range, there are two centers of high diversity for *Rhododendron* that reflects diversification events. (Figure 1.3)

One center of diversity is located in western China (S in Figure 1.3) in Yunnan province where more than 500 species have been identified (Feng 1992). The high diversity of *Rhododendron* in Yunnan province is most likely due to a varied mountain relief with many isolated valleys and a complex geological history that resulted from the collision of the Indian and Asian continental plates.

A second center of diversity is in the tropical archipelago of Malesia (A-Q in Figure 1.3), which is the area between Southeast Asia and Australia and includes Malaysia, Indonesia, Philippines, Papua and New Guinea. The Malesian area is composed of three plates; Pacific, Indo-Australian, and Indo-China (Michaux 1991). The diversity of topography created by the nexus of these three plates has created a diversity of habitats and a hot spot for biological diversity for many taxa including *Rhododendron*. *Rhododendron* species are widely spread throughout the archipelago (Brown et al. 2006). Most *Rhododendron* species (287 out of 297) in the Malesian area are classified within subgenus *Vireya*, which have diversified into this area after SE Asia-Malesia contacted the Indo-Australian plate (20 million YBP). However, diversification into this region most likely took place during the past 5 million years (Stevens 1985). The main characteristics of species in subgenus *Vireya* are two-tailed wind-dispersed seeds, a twisted capsule valve when opened, thread-like placentas and idioblast cells below the adaxial epidermis (Argent 2015).

Brown et al. (2006) proposed two possibilities for the origin of subgenus *Vireya*. The first hypothesis is that subgenus *Vireya* is an old group and its ancestors were in Gondwanan and diversified in Malesia from the Indo-Australian plate. Another hypothesis is that subgenus *Vireya* is a younger group, which diversified into Malesia from the Indo-China plate moving south as Malesia was formed (Brown et al. 2006). Based on newly reported molecular evidence for subgenus *Vireya* phylogeography, it is clear that this group is derived from a northern group (Indo-China ancestral types) and diversified into the Malesian archipelago (Goetsch et al. 2011).

1.33 Temperate to tropical diversification— Subgenus *Vireya*, a monophyletic group, contains seven well-supported monophyletic sub groups (Figure 1.4) as defined by molecular phylogenetic analyses (Brown et al 2006). These seven groups are based on similarities in the sequence of cpDNA, but they also have a geographic pattern. In fact, the basal groups are northerly and the derived groups have a southerly distribution. This clear diversification pattern becomes an ideal system for studying how functional traits in this group changed during this diversification event from temperate habitats into tropical regions (Figure 1.4).

1.34 Relict distributions—There is strong evidence that some of the diversity in *Rhododendron* is derived from relict distributions (small range that survives from much larger range from the past). The best evidence to support relict distributions is for subgenus *Hymenanthes* (Milne 2004). *Ponticum* is the only section in subgenus *Hymenanthes*. Of the 24 subsections in section *Ponticum*, subsection *Pontica* is the largest and best-known subsection and probably the oldest group within *Hymenanthes*. There is a high possibility that *Pontica* diverged and existed before or during the

distribution of the group in the circumboreal forest of Laurasia, rather than afterward. Subsection *Pontica* is composed of 11 species distributed throughout the northern hemisphere in temperate mountain habitats. Four species are native to Turkey and surrounding areas (*R. caucasicum*, *R. smirnowii*, *R. ungerii*, and *R. ponticum*), 2 species are found in eastern North America (*R. maximum* and *R. catawbiense*), 1 species is native to western North America (*R. macrophyllum*), and 3 species (*R. degonianum*, *R. brachycarpum*, and *R. aureum*) are native to northern Asia, including Japan, Korea, and Taiwan (Milne 2004). The distribution pattern of *Hymenanthus* changed from wide range throughout northern hemisphere circa 50 million years ago to the currently more restricted area mainly because of climate fluctuation and geographic change, i.e. continental drift (Irving 1993, Milne and Abbott 2002). Since my study concerns the anatomical traits in temperate and tropical species, the focus of my research is on diversification from temperate mountain habitat into tropical mountain habitats, although some taxa from subgenus *Hymenanthus* were included.

1.4 Current knowledge of *Rhododendron* anatomical traits

This research project focuses on leaf and stem anatomical traits of *Rhododendron*. Many temperate *Rhododendron* species have thermonastic leaf movement (TLM) under freezing temperature. The description of TLM is that leaf angle drops at temperature close to 0 °C and leaf rolling starts rapidly at -2 °C and progresses during further temperature decrease (Nilsen 1992). It has been proven that thermonastic leaf movement serves to prevent photoinhibition during winter freezes (Russell et al. 2009) and results in

freezing tolerance. Leaf thickness and palisade mesophyll can be a key to plant adaptation to avoid light injury during unfavorable conditions. *R. catawbiense* is one example that possibly copes with winter light and water problems with thermonastic leaf movement (Wang et al. 2008). It has been suggested that leaf anatomical structures are associated with physiological functionality in *Rhododendron* and provide more competitiveness for each species in different environments (Cai et al. 2014). Leaf scales were observed in many species of *Rhododendron*, especially in the subgenus *Rhododendron*. The scales vary in size, shape and density. It has been suggested that leaf scales of *Rhododendron* help with water conservation through stomata density interference (Nilsen et al. 2014).

Stem vessel size is associated with hydraulic conductivity (efficiency). Smaller vessel diameters provide more resistance to freeze-thaw or drought (safety), but at the same time, reduce hydraulic conductance (efficiency). Temperate *Rhododendron* species experiencing more frequent freeze-thaws had smaller vessel diameters than temperate *Rhododendron* species experiencing fewer freeze-thaw events (Cordero and Nilsen 2002). Therefore, xylem safety is an important trait in *Rhododendron* for tolerating freeze-thaw cycles (Lipp and Nilsen 1997, Cordero and Nilsen 2002).

One goal of this study is to understand the change in anatomical traits that occurred as plants diversified from a temperate region into a tropical region. The dramatic difference in climate encountered by plants diversifying from temperate origins into tropical climates ought to result in predictable changes in anatomical traits based on our knowledge of the functional significance of those anatomical traits to plant fitness. In temperate regions, the weather strongly fluctuates among seasons of the year compared to

seasonal weather changes in tropical regions (Figure 1.5) including frequent freeze events during the winter (Lipp and Nilsen 1997). In tropical mountain habitats, daily temperature variation greatly exceeds seasonal variation in temperature and, except at very high-altitude tropical mountain habitats, freezing does not occur. The frequency of freeze-thaw events increases with an increase in latitude and with an increase in elevation (Lipp and Nilsen 1997). Therefore, the work focus on anatomical trait association with latitude (temperate vs. tropical) and elevation of the native range.

*1.41 Temperate *Rhododendron* anatomical traits—Temperate evergreen*

Rhododendron species must be well adapted to cope with large seasonal changes in temperature if they are to maintain their leaves over the winter. The most fatal damage caused by harsh condition during the winter in temperate regions is tissue and vascular damage by freezing. High radiation, at low winter temperature can induce buildup of reactive oxygen species in leaves, which will have a negative effect on photosynthesis the next spring. Also, water availability can become a problem during winter in temperate zones because cold soil limits water availability and the ability of roots to extract water from the soil.

There are several anatomical traits of *Rhododendron* that are suggested to be adaptive to temperate conditions. The 3 most important are thermonastic leaf movements (Nilsen 1992), osmotic adjustment, and narrow vessel diameter (Cordero and Nilsen 2002). Thermonastic leaf movements have been shown to protect chloroplast integrity (Bao and Nilsen 1988) and photosynthetic performance in the early spring (Russell et al. 2009). Osmotic adjustment allows for the maintenance of turgor pressure at low bulk leaf water potential. Also, during freezing temperature, the cell cytosol must remain liquid.

Ice crystals in the cell can break the nucleus and cell membrane thereby damaging important plant organs. Osmotic adjustment can prevent large ice crystal formation by reducing the leaf freezing point, thereby decreasing the likelihood of freeze-thaw induced cell damage. Narrow vessels have been shown to prevent freeze-thaw induced embolism (Cordero and Nilsen 2002). However, the narrow vessels reduce the capacity of the stem to carry water during the main growing season, which constrains overall growth rate. As a result, temperate *Rhododendron* grow relatively slowly and are sensitive to drought, yet they are one of the few temperate evergreen shrubs with freeze-thaw tolerance. Narrow vessels would be a detriment to growth in a tropical environment and this ought to reduce the capacity of *Rhododendron* plants to compete with plants that have higher growth rates in tropical environments. In addition, osmotic adjustment, an energy expensive process, would not be valuable for tropical environments with ample water availability and no freeze-thaw events. (Figure 1.5)

1.42 Tropical Rhododendron anatomical traits—In contrast, tropical environments in the Malesian region are characterized by small seasonal variation in temperature and soil water availability compared with temperate zone climates. Tropical mountain habitats, which are home for many tropical *Rhododendron* species, are characterized by small seasonal variation in temperature and rare freeze-thaws. Also, tropical mountain habitats can have high humidity and rainfall because of humidified sea air moving up in elevation. However, some locations in the Malesian tropics can have wet and dry seasons.

The warmer temperature, fewer freeze-thaw events and higher water availability of tropical Malesian environments ought to favor larger vessels than in temperate

environments because of the small risk for freeze-induced vascular cavitation. However, narrow vessels can result in a constraint on productivity. Moreover, if tropical plants experience drought, either due to a dry season or high demand for water flow, then drought-induced embolism can become a problem. Therefore, natural selection processes should favor plants with wider vessels in tropical environments. Moreover, osmotic adjustment to lower leaf freezing point has a metabolic cost. Therefore, species without osmotic adjustment in the winter season would have a selective advantage in tropical climates compared to those who use osmotic adjustment in response to cool temperatures. Thermonastic leaf movements would provide no selective advantage for species in tropical environments; however, there may not be any metabolic cost of thermonastic leaf movements and the movements may be a left over from the temperate origin of the tropical species.

Tropical *Rhododendron* species can be epiphytic, while no temperate *Rhododendron* species are epiphytic. An epiphytic habit results in unique water management problems. The medium, organic matter on a branch, does not retain water for long periods. Therefore, epiphytes are likely to experience frequent short duration drought. This is one reason why many epiphytes or succulent (orchids, ferns, stonecrops), use CAM photosynthesis or have leaf architectural mechanisms for storing water (bromeliads). Tropical epiphytic *Rhododendron* species exclusively use C3 photosynthesis, do not have architecture conducive for storing water, but may have anatomic mechanisms for buffering water balance with succulence such as thick leaves with high water content. Large cells found just below the adaxial epidermis in some

tropical *Rhododendron* species called idioblast may serve as a water buffer system, particularly for species that have an epiphytic growth habitat.

1.5 Overall goal of this work

The goal of this research is to discover plant leaf and stem anatomical traits that are associated with adaptation of *Rhododendron* to temperate or tropical environments. *Rhododendron* is one of a few plant genera that are distributed globally in both tropical and temperate regions. The closely-related groups of *Rhododendron* with similar leaf morphology were selected for this study to reduce variation in genetic history and thus reveal environment–associated adaptive traits

1.51 Approach Taken in this research effort—A survey approach was used to address hypotheses about the relationships between wood or leaf anatomical traits and habitat.

1) Wood anatomical traits in more than 60 species of *Rhododendron* were measured. The effects of habitat type (temperate vs. tropical) and elevation were tested with two-way ANOVA and regression.

2) Leaf anatomical traits among 80 accessions of *Rhododendron* were measured. The effects of habitat type (temperate vs. tropical) and elevation were tested with two-way ANOVA and regression.

3) Adaxial idioblast expression and leaf water relation traits were measured in 61 plants covering 17 species of *Rhododendron*. The relationships between

idioblast expression (and other leaf anatomical traits) and plant water relation traits were evaluated with regression and stepwise analysis.

4) Overall patterns in leaf and wood anatomical traits in *Rhododendron* were summarized. The relationship with habitat (temperate vs. tropical) and elevation was considered in light of *Rhododendron* ecology and plant water relations.

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1.7 Tables

Table 1.1 *Rhododendron* subgenera and sections defined by Chamberlain and Royal

Botanic Garden (1996)

Subgenus	Section	Number of Species
<i>Rhododendron</i>	<i>Pogonanthum</i>	21
	<i>Rhododendron</i>	211
	<i>Vireya</i>	310
<i>Hymenanthus</i>	<i>Ponticum</i>	302
<i>Tsutsusi</i>	<i>Brachycalyx</i>	23
	<i>Tsutsusi</i>	94
<i>Pentanthus</i>	<i>Pentanthus</i>	23
	<i>Rhodora</i>	2
	<i>Sciadorhodion</i>	4
	<i>Viscidula</i>	1
<i>Azaleastrum</i>	<i>Azaleastrum</i>	11
	<i>Choniastrum</i>	19
<i>Therorhodion</i>	-	2
<i>Mumeazalea</i>	-	2
<i>Candidastrum</i>	-	2

Table 1.2 The classification of *Rhododendron* based on the 2005 treatment by Goetsch et al. (2005)

Subgenus	Section
<i>Rhododendron</i>	<i>Pogonanthum</i>
<i>Rhododendron</i>	<i>Rhododendron</i>
<i>Rhododendron</i>	<i>Vireya</i>
<i>Hymenanthus</i>	<i>Ponticum</i>
<i>Hymenanthus</i>	<i>Penthanthera</i>
Tsutsusi	<i>Brachycalyx</i>
Pentanthera	<i>Rhodora</i>
<i>Azaleastrum</i>	<i>Tsutsusi</i>
<i>Azaleastrum</i>	<i>Sciadorhodion</i>
<i>Therorhodion</i>	
<i>Choniastrum</i>	

Table 1.3 The two most recent classifications of *Rhododendron* section *Schistanthe* (Craven et al. 2011, Goetsch et al. 2011) in comparison with subgenus *Vireya* (Argent 2015)

Craven et al. 2011			Argent 2015		
Subgenus	Section	Subsection	Subgenus	Section	Subsection
<i>Rhododendron</i>	<i>Schistanthe</i>	<i>Discovireya</i>	<i>Vireya</i>	<i>Albovireya</i>	
		<i>Euvireya</i>		<i>Discovireya</i>	
		<i>Malayovireya</i>		<i>Hadranthe</i>	
		<i>Pseudovireya</i>		<i>Malayovireya</i>	
				<i>Pseudovireya</i>	
				<i>Schistanthe</i>	<i>Euvireya</i>
				<i>Schistanthe</i>	<i>Linnaeopsis</i>
				<i>Schistanthe</i>	<i>Malesia</i>
				<i>Schistanthe</i>	<i>Saxifragoidea</i>
				<i>Schistanthe</i>	<i>Solenovireya</i>
				<i>Siphonovireya</i>	

1.8 Figure legends

Figure 1.1 Comparison diagram of Leaf Economic Spectrum (LES) traits between fast-growing and slow-growing plants modified from Royer (2008)

Figure 1.2 A diagrammatic representation of the maximum parsimony strict consensus tree based on *RPB2-I* gene adapted from Goetsch et al. (2005). The number represent bootstrap value.

Figure 1.3 Focus areas for *Rhododendron* species diversity. Adapted from Feng (1992). Blue circle indicate the range of temperate *Rhododendron* species used in this study. Red circle indicate the range of tropical *Rhododendron* species used in this study.

Figure 1.4 The geographic distribution of the 7 monophyletic groups of *Vireya*. Adapted from Goetsch et al. (2011).

Figure 1.5 Seasonal and daily patterns of general climatic conditions in temperate and tropical mountain habitats. Solid blue lines refer to seasonal mean temperatures and dashed lines represent the daily variation in temperature. Solid black line refers to 0 °C.

1.9 Figures

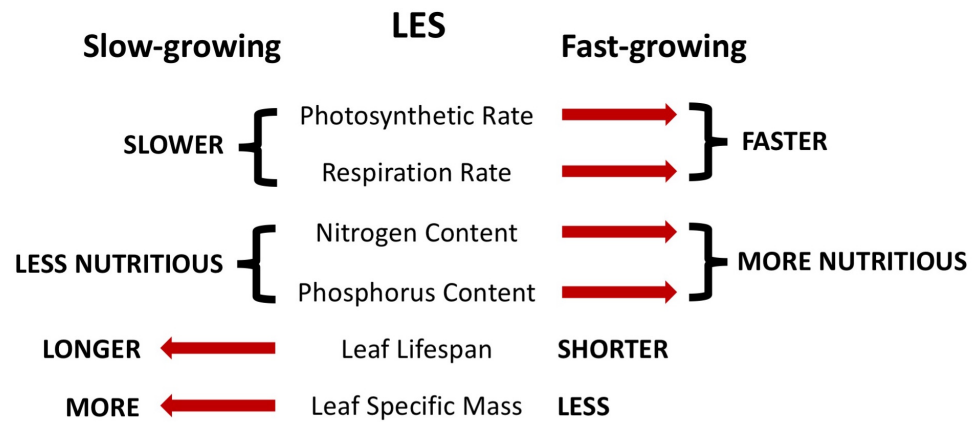


Figure 1.1. Comparison diagram of Leaf Economic Spectrum

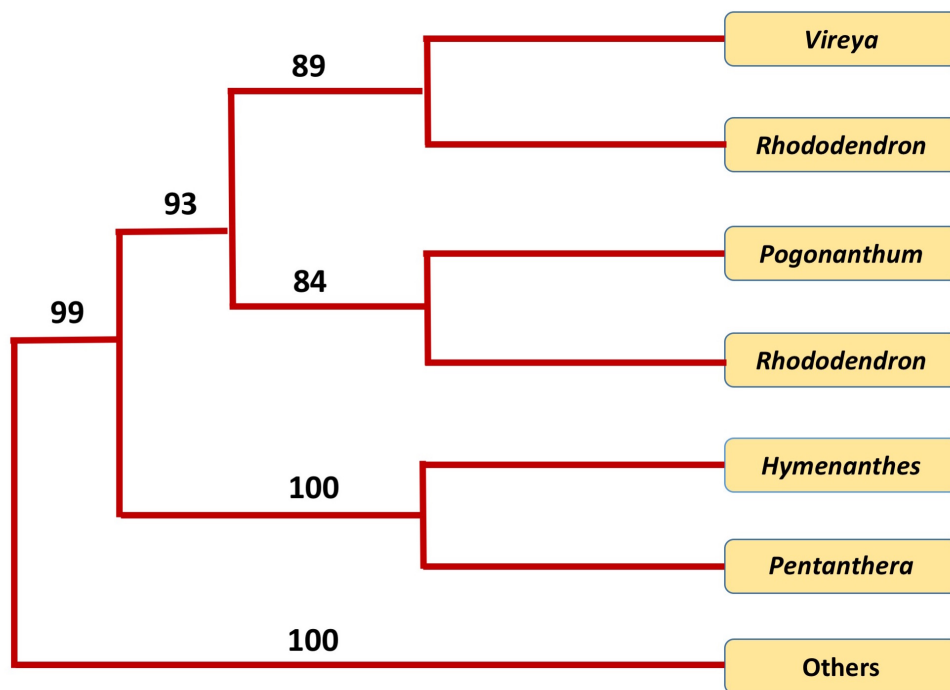


Figure 1.2. A diagrammatic representation of consensus tree based on *RPB2-I* gene



Figure 1.3. Focus areas for *Rhododendron* species diversity



Figure 1.4. The geographic distribution of the 7 groups of *Vireya*

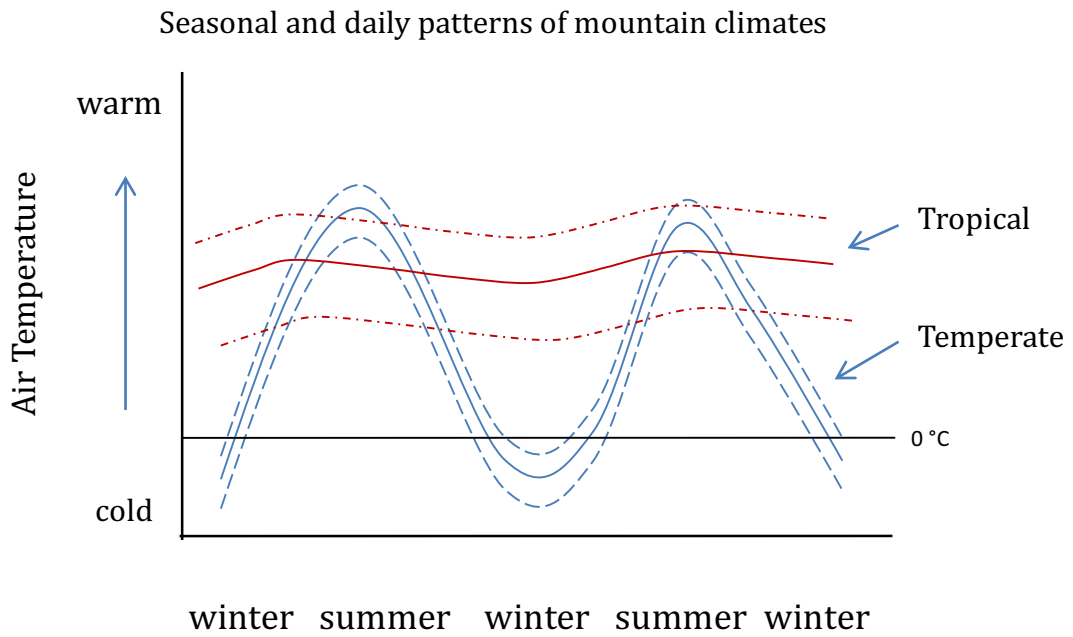


Figure 1.5. Climatic conditions in temperate and tropical mountain habitats

CHAPTER 2

Exploration of vascular anatomical traits among species of *Rhododendron*

2.1 Introduction

The structure of plant vascular tissue is a major selective trait for plant distribution and drought tolerance (Anderegg et al. 2016) (Figure 2.1). Larger vessel diameters lead to more efficient water flow (Sperry et al. 2006), but at the same time, render the plant more susceptible to vessel embolism after freeze-thaw events (Hargrave et al. 1994, Cordero and Nilsen 2002). Smaller vessel diameters increase safety against freeze-thaw induced embolism during the winter (Lens et al. 2013), however, it will constrain plant productivity due to water flow restriction during the summer. The result is a potential tradeoff between safety and efficiency of wood (Meinzer et al. 2010). Temperate plants, or plants at high elevation sites, which will likely face many freeze-thaw events during the winter, are expected to favor safety over efficiency in vascular traits. Although there was a lot of support for the trade-off, many studies did not find the trade-off (Gleason et al. 2016).

There are many characteristics of vessels that influence safety. As stated above smaller vessels are less likely to embolize due to freezing and may be easier to refill after freezing (Lens et al. 2013). The characteristics of vessel diameter distribution are also important for safety. For example, a smaller proportion of vessels with relatively large diameters promotes safety. Thus, the more positive the skewness (the proportion of the data sample on one versus the other side of the mean) of the vessel diameter distribution the safer the vascular system (Medeiros and Pockman 2014). A homogeneous vessel

distribution (high kurtosis; the extent of data spread around the mean) are more likely to suffer catastrophic vascular failure than a heterogeneous distribution (lower kurtosis). Other elements of vessel safety are a low number of vessel pits, a low connectivity between adjacent vessels and relatively thick cell walls.

Similarly, there are many facets of vascular efficiency. Primary among these is a relatively high water transport capacity. High water transport capacity (efficiency) can be attained by a larger mean hydraulically weight vessel diameter, more connectivity among vessels or a higher vessel density (Meinzer et al. 2010). Thus, plants with high efficiency have a high theoretical specific conductivity, a higher proportion of vessels in high diameter size classes, higher kurtosis and less negative skewness.

If temperate evergreen plants favor safety over efficiency due to a high frequency of freeze-thaw events, then tropical plants ought to favor efficiency due to a low frequency of freeze-thaw events. Moreover, a fast growth rate may be a competitive advantage in a wet-tropical environment. Fast growth for evergreen plants is due to a high leaf area ratio and an efficient vascular system (Meinzer et al. 2010). Thus, the vascular system of evergreen shrubs in a wet-tropical habitat should favor efficiency rather than safety. However, in seasonally dry tropical habitats or higher elevation tropical habitats the balance of efficiency to safety may be tipped toward safety. Therefore, the balance of efficiency and safety may be strongly influenced by both elevation and habitat type.

There are three main ways to determine the functional significance of traits to different habitats. One possible approach is to broadly select plant samples from many different families to make sure that the selection covers as many groups as possible and is

representative of each habitat under consideration. Another way is to utilize the comparative method based on a known and well established phylogeny. A third way is to study these traits in one narrow closely-related monophyletic taxonomic group that exists across all habitat types. This study focuses on the functional significance of vascular traits across habitat types in a closely-related monophyletic group of plants.

Subgenus *Rhododendron* is a well-supported monophyletic clade (93% bootstrap; Goetsch et al. 2005) in the genus *Rhododendron* is a good candidate for this research. The species in this subgenus can be found world-wide in many northern hemisphere temperate habitats and in tropical regions including Thailand, Malaysia, Indonesia, Taiwan, Papua, and New Guinea. Moreover, all the species in this group are evergreen shrubs or small trees (Argent 2015). In addition, there are two major collections of living *Rhododendron* germplasm available for research purposes: (1) The *Rhododendron* Species Foundation and Botanical Garden, Federal Way, WA, and (2) The Royal Botanic Garden Edinburgh, UK.

The overall goal of this research was to determine if the balance of safety and efficiency in stems shifts from an emphasis on safety in temperate *Rhododendron* species to an emphasis on efficiency in tropical *Rhododendron* species. The hypotheses addressed in this study were:

1) The wood of temperate *Rhododendron* species is significantly safer than the wood of tropical species of *Rhododendron*. This hypothesis would be supported if: a) The mean vessel diameter for temperate *Rhododendron* species is significantly smaller than the tropical counterpart, b) The vessel diameter distribution for temperate *Rhododendron* species is skewed toward smaller size than the tropical species, c) The kurtosis of vessel

diameter distribution in temperate *Rhododendron* species is significantly smaller than the tropical species.

2) The wood of *Rhododendron* species is significantly safer for those species that have a higher mean elevation of their native range compare with species that have a lower mean elevation of their native range. This hypothesis would be supported if: a) Species with a high native elevation range have a significantly smaller mean vessel diameter than species with a medium or low mean elevation of the native range, b) Species with a high native elevation range have a vessel distribution skewed toward smaller size than species with a medium or low mean elevation of the native range, c) The vessel distribution for species with a high native elevation range has a significantly smaller kurtosis than species with a medium or low mean elevation of the native range.

3) The wood of *Rhododendron* species that have a tropical range has significantly higher transport efficiency than the wood of *Rhododendron* species that have a temperate range. This hypothesis would be supported if: a) The vessels of tropical *Rhododendron* species have a significantly larger mean hydraulically weighted diameter (D_h) than that of temperate *Rhododendron* species, b) Tropical *Rhododendron* species have a significantly higher vessel density (pV) than that of temperate *Rhododendron* species, c) Tropical *Rhododendron* species have a significantly higher theoretical specific conductivity (K_s^{theo}) than that of temperate *Rhododendron* species. In addition, the vessel diameter distribution of tropical species will be skewed toward larger size, while kurtosis is more positive.

All accession used in this study were from Rhododendron Species Foundation and Botanical Garden in Federal Way, WA, a common garden in Volcano Village, HI, the

Royal Botanic Garden Edinburgh, UK, and Biological Sciences-VBI Plant Growth Facility (VT-PGF) (Table 2.1). Vessel traits critical to both safety and efficiency were measured on all specimens. Analysis of variance (ANOVA), and regression were used to find significant effects of habitat (temperate vs. tropical) and native range elevation on vascular traits.

2.2 Materials and methods

2.21 Selected accessions—Approximately 60 accessions of *Rhododendron* were selected for this research (Table 2.1). The samples include evergreen shrubs that have temperate or tropical ranges. Wood of accessions was sampled from various sources including the Rhododendron Species Foundation and Botanical Garden, Federal Way, WA, a common garden in Volcano Village, HI, the Royal Botanic Garden Edinburgh, UK, and Biological Sciences-VBI Plant Growth Facility (VT-PGF). One stem was sampled from each accession.

2.22 Stem preparation and microtomy—Young stems (at least 1.0 cm in diameter and 20-30 cm long) were excised from the outer canopy of each accession and carefully labeled. The excised stems were sealed in plastic bags prior to transport to the ecological physiology lab at Virginia Tech. Upon arrival to the lab, the stems were promptly refrigerated (4 °C). A 1.5 cm segment was cut from each end of the stem section in order to have two independent assays of vessel from each stem. Previous research has shown that *Rhododendron* vessels elements are less than 3 cm in length, so the selection of segments at each side of the stem does not include the same vessels. Stem cross-sections between 15-20 μm thick were made from each end of the stem using lightweight slide

microtome (WSL GSL-1, Birmensdorf, Switzerland) (Gartner et al. 2014). Cross sections were maintained in distilled water until dehydration and staining was done.

2.23 Staining and dehydration—Specimens were gently picked from the distilled water with a paintbrush and put on metal meshes. A drop of safranin O solution was applied to the specimen for about 3 seconds before gently flushing with distilled water three times. Subsequently the samples were dehydrated by sequentially flushing with an increasing ethanol gradient (50%, 75% 100%) for 5 s each flush followed by a 5 s flush with toluene. Wet specimens were placed on a pre-cleaned microscopic slide. The resulting specimens were fixed in Permounttm for later vessel measurements.

2.24 Xylem traits measured—Nikon NIS-Element Basic Research version 4 and Nikon Eclipse LV100 with digital camera head DS-Fi1 (Nikon Inc., Tokyo, Japan) were used to observe and to make measurement of the specimen. The measurements were conducted at 40 x objective lens. The data were exported through Nikon NIS-Element Basic Research to a spreadsheet for further analysis. The diameter and area of every vessel in 10 fields of view were determined resulting in about 75 - 125 vessels assayed on each side of the stem. The total number of vessels measured in the total area of stem ($\# / \text{mm}^2$) was considered the vessel density (pV). The hydraulically weighted diameter was calculated as: $D_h = \Sigma D^5 / \Sigma D^4$ where D represents vessel diameter (μm) (Sperry and Saliendra 1994b). The theoretical specific conductivity (K_s^{theo}) was calculated by $((\pi \times \Sigma D^4) / 128 \times \eta) \times \rho / \text{vascular area (mm}^2\text{)}$ for each sample where η represents the viscosity of water (1.002×10^{-9}) and ρ represents the density of water (998.2 kg m^{-3}). The units for this trait is $\text{kg m}^{-1} \text{ MPa}^{-1} \text{ s}^{-1}$. Kurtosis and skewness of the data was calculated for each stem. Kurtosis was interpreted as the evenness of investment in water transport capacity

across all vessel diameters. Relatively high skewness was interpreted as relative high investment in the smallest (less vulnerable to cavitation) compared with the largest (more vulnerable to cavitation) vessels. Relative vulnerability to freeze-thaw embolism was determined as in Medeiros and Pockman (2014). Vessel diameter never exceeded 44 μm ; therefore, the cutoff diameter suggested by Davis et al. (1999) and used by Medeiros and Pockman (2014) cannot be used. Instead, the vessel diameter at 24 μm was chosen as a reference value (based on personal observation) for calculating relative vulnerability to freeze-thaw induced embolism. Therefore, the index of relative vulnerability is the proportion of total water transport accounted for by vessels greater than 24 μm in diameter. This is a very conservative determination of relative vulnerability to embolism in comparison to other taxa previously studied.

After the cortex was removed, the basal area of the each sampled stem was determined as well as the area of pith. The vascular area was calculated as the stem area minus the pith area.

2.25 Statistical analysis—Accessions were assigned to a habitat (temperate or tropical) based on their known native range reported in the literature. Tropical species were those that had ranges between 20 °N and 20 °S latitude. Temperate species had ranges between 30 °N and 40 °N latitude. An elevation was assigned to each species as the middle elevation of the native range reported in the literature. In order to convert these elevation data from continuous to categorical three categories were defined (low = 0 - 1,200 m, medium = 1,201 - 2,400 m, high = 2,401 - 4,200 m). Two-way ANOVA was used to test the effects of habitat (temperate, tropical), elevation (low, medium, and high) and their interaction on all vascular traits (JMP Pro 12, SAS Institute Inc., Cary, NC).

The effect of either stem area or vascular area (continuous variable) on vessel traits was determined for stems from each habitat by regression analysis. A significant regression slope was considered a significant effect of stem or vascular area on vessel traits. The difference in regression slopes was used to determine if there was a different relationship between stem or vascular area and vessel traits for temperate or tropical stems. A similar regression technique was used to determine the effect of the mean elevation of the native range on the vascular traits.

2.3 Results

2.3.1 ANOVA results—The mean and standard deviation of vessel diameter (Figure 2.2 A) of all data is $16.55 \mu\text{m} \pm 2.95$. The mean vessel diameter of tropical plants ($18.29 \mu\text{m} \pm 3.27$) was significantly higher (Table 2.2) than the temperate species ($15.47 \mu\text{m} \pm 2.15$). The mean vessel diameter tended to decrease from low elevation to high elevation ($18.22 \mu\text{m} \pm 3.87$, $16.99 \mu\text{m} \pm 2.94$, $15.54 \mu\text{m} \pm 2.34$ respectively) but the elevation effect was not significant (Table 2.2). The interaction between habitat and elevation was not significant. The mean and standard deviation of minimum vessel diameter (Figure 2.2 B) of all data was $10.25 \mu\text{m} \pm 1.79$. The tropical plants had a significantly (Table 2.2) higher minimum vessel diameter ($10.47 \mu\text{m} \pm 2.16$) than that of the temperate plants ($10.11 \mu\text{m} \pm 1.54$). The minimum vessel diameter significantly increased (Table 2.2) from the low to the high elevation ranks ($9.77 \mu\text{m} \pm 2.59$, $10.07 \mu\text{m} \pm 1.63$, $10.60 \mu\text{m} \pm 1.69$, respectively). The interaction between habitat and elevation was not significant. The mean and standard deviation of maximum vessel diameter (Figure 2.2 C) of all data was $24.41 \mu\text{m} \pm 4.89$. The tropical plants had a significantly (Table 2.2)

higher maximum vessel diameter ($26.71 \mu\text{m} \pm 5.23$) than that of the temperate plants ($22.98 \mu\text{m} \pm 4.13$). The maximum vessel diameter tended to decrease from low elevation to high elevation ($27.48 \mu\text{m} \pm 5.30$, $25.24 \mu\text{m} \pm 4.99$, $22.54 \mu\text{m} \pm 4.01$ respectively) but the elevation effect was not significant (Table 2.2). The interaction between habitat and elevation on maximum vessel diameter was not significant. The mean and standard deviation (Figure 2.2 D) of vessel density (pV) of all data is $991.78 \mu\text{m} \pm 219.85$. The mean vessel density of temperate plants ($1044.76 \text{mm}^{-2} \pm 198.30$) tended to be higher than the tropical species ($906.55 \text{mm}^{-2} \pm 230.16$), but the trend was not statistically significant (Table 2.2). The mean vessel density tended to increase from low, and medium elevation to high elevation ($956.16 \text{mm}^{-2} \pm 129.04$, $943.03 \text{mm}^{-2} \pm 231.30$, $1055.83 \text{mm}^{-2} \pm 220.55$ respectively) but the elevation effect was not significant (Table 2.2). The interaction between habitat and elevation was not significant.

The mean and standard deviation of all vessel diameter distribution skewness (Figure 2.3 A) was 0.289 ± 0.26 . The temperate plants had a significantly (Table 2.3) higher skewed distribution (0.386 ± 0.221) than that of the tropical plants (0.132 ± 0.256). The skewness of the distribution significantly (Table 2.3) increased from the low to the high elevation ranks (0.166 ± 0.291 , 0.243 ± 0.280 , 0.392 ± 0.196 , respectively). The interaction between habitat and elevation was not significant for skewness. The mean and standard deviation of all vessel diameter distribution kurtosis (Figure 2.3 B) was 0.360 ± 0.59 . The tropical plants tended to have higher vessel diameter distribution kurtosis (0.438 ± 0.613) than the temperate plants (0.311 ± 0.572), but that trend was not statistically significant (Table 2.3). The mean distribution kurtosis tended to decrease from low to high elevation (0.508 ± 0.440 , 0.403 ± 0.608 , 0.266 ± 0.608 respectively),

but the elevation effect was not significant (Table 2.3). The interaction between habitat and elevation was not significant.

The mean and standard deviation of the hydraulically weighted diameter of xylem (D_h) (Figure 2.4 A) in all data is $17.78 \mu\text{m} \pm 3.20$. The mean D_h of tropical plants ($19.64 \mu\text{m} \pm 3.55$) was significantly higher (Table 2.4) than the temperate species ($16.62 \mu\text{m} \pm 2.34$). The mean D_h tended to decrease from low elevation to high elevation ($19.65 \mu\text{m} \pm 4.16$, $18.31 \mu\text{m} \pm 3.20$, $16.61 \mu\text{m} \pm 2.48$ respectively), but the elevation effect was not significant (Table 2.4). The interaction between habitat and elevation was not significant.

The mean and standard deviation of the theoretical specific conductivity (Figure 2.4 B) in all data was 2.09 ± 1.09 . The mean theoretical specific conductivity of tropical plants ($2.51 \pm 1.20 \text{ kg m}^{-1} \text{ MPa}^{-1} \text{ s}^{-1}$) was significantly higher (Table 2.4) than the temperate species ($1.87 \pm 0.98 \text{ kg m}^{-1} \text{ MPa}^{-1} \text{ s}^{-1}$). The mean theoretical specific conductivity tended to decrease from low elevation to high elevation (2.50 ± 1.10 , 2.19 ± 1.11 , $1.89 \pm 1.08 \text{ kg m}^{-1} \text{ MPa}^{-1} \text{ s}^{-1}$ respectively), but the elevation effect was not significant (Table 2.4).

The interaction between habitat and elevation was significant, therefore the effect of elevation on theoretical specific conductivity was different for the two habitat types. The mean and standard deviation of the proportion of theoretical transport in vessel diameter larger than $24.00 \mu\text{m}$ ($\Sigma D^4 > 24$) of all data (Figure 2.4 C) was $7.10 \pm 14.15 \%$. The tropical plants had a significantly (Table 2.4) higher $\Sigma D^4 > 24$ ($14.52 \pm 20.19 \%$) than that of the temperate plants ($2.50 \pm 4.67 \%$). The $\Sigma D^4 > 24$ significantly (Table 2.4) decreased from the low to the high elevation ranks (16.53 ± 27.37 , 8.93 ± 13.51 , 2.12 ± 4.10 , respectively). The interaction between habitat and elevation was not significant.

2.32 Regression results—The regression of mean vessel diameter (dependent variable) against average elevation of the native range (independent variable) was statistically significant (Table 2.5) when all species were included ($R^2 = 0.144$, negative slope), and when only temperate species were included ($R^2 = 0.079$, negative slope). However, this regression was not statistically significant when only tropical species were included ($R^2 = 0.032$, negative slope). The regression of minimum vessel diameter against average elevation of the native range was not statistically significant when all species were included ($R^2 = 0.013$, positive slope), or when only temperate species ($R^2 = 0.018$, positive slope) were included (Table 2.5). This regression was statistically significant ($R^2 = 0.140$, positive slope) when only tropical species were. The regression of maximum vessel diameter against average elevation of the native range was statistically significant (Table 2.5) when all species were included ($R^2 = 0.167$, negative slope), and when only temperate species were included ($R^2 = 0.176$, negative slope). However, this regression was not significant ($R^2 = 0.002$, negative slope) when only tropical species were included. The regression of vessel density (pV) against average elevation of the native range was statistically significant (Table 2.5) when all species were included ($R^2 = 0.062$, positive slope). This regression was not statistically significant when either only temperate species ($R^2 = 0.009$, positive slope) or only tropical species ($R^2 = 0.085$, positive slope) were included (Table 2.5).

The regression of vessel diameter distribution skewness against average elevation of the native range was statistically significant when all species were included ($R^2 = 0.136$, positive slope), and when only tropical species were included ($R^2 = 0.313$, positive slope). However, this regression ($R^2 = 0.011$, positive slope) was not statistically

significant (Table 2.5) when only temperate species were included. The regression of vessel diameter distribution kurtosis against average elevation of the native range was not statistically significant (Table 2.5) when all species were included ($R^2 = 0.032$, negative slope), only temperate species were included ($R^2 = 0.069$, negative slope), or when only tropical species ($R^2 = 0.036$, positive slope), were included.

The regression of hydraulically weighted diameter of xylem (D_h) against average elevation of the native range was statistically significant when all species are included ($R^2 = 0.159$, negative slope), and when only temperate species are included ($R^2 = 0.099$, negative slope). This regression was not statistically significant when only tropical species ($R^2 = 0.040$, negative slope), were included (Table 2.5). The regression of theoretical specific conductivity (K_s^{theo}) against average elevation of the native range was statistically significant when all species were included ($R^2 = 0.070$, negative slope), and when only temperate species were included ($R^2 = 0.088$, negative slope). This regression was not statistically significant (Table 2.5) when only tropical species were included ($R^2 = 0.043$, positive slope). The regression of the proportion of theoretical transport in vessel with larger diameter ($> 24.00 \mu\text{m}$) ($\Sigma D^4 > 24$) against average elevation of the native range was statistically significant (Table 2.5) when all species were included ($R^2 = 0.142$, negative slope), when only temperate species were included ($R^2 = 0.153$, negative slope), and when only tropical species were included ($R^2 = 0.161$, negative slope).

The regression of mean vessel diameter (as dependent variable) against vessel density (as independent variable) was statistically significant (Figure 2.5 A) with a negative slope when all species were included ($p\text{-value} = 0.0625$, $R^2 = 0.059$). Although this regression had a negative slope the regression was not significant when only

temperate (p -value = 0.54, R^2 = 0.010) or only tropical species (p -value = 0.552, R^2 = 0.017) were included (Figure 2.5 B, 2.5 C).

The regression of vascular area (as dependent variable) against whole stem cross section area (as independent variable) was statistically significant (p -value < 0.0001, R^2 = 0.950) when all species were included (Figure 2.6 A), when only temperate species were included (Figure 2.6 B, p -value < 0.0001, R^2 = 0.937), and when only tropical species were included (Figure 2.6 C, p -value < 0.0001, R^2 = 0.943). Therefore, vascular area scaled linearly with stem area.

The regression of mean vessel diameter against vascular area was statistically significant (Table 2.6) when all species were included (R^2 = 0.368, positive slope), when only temperate species were included (R^2 = 0.182, positive slope), and when only tropical species were included (R^2 = 0.334, positive slope). The regression of minimum vessel diameter against vascular area was not statistically significant (Table 2.6) when all species were included (R^2 = 0.027, positive slope), when only temperate species were included (R^2 = 0.019, positive slope), or when only tropical species were included (R^2 = 0.017, positive slope). The regression of maximum vessel diameter against vascular area was statistically significant (Table 2.6) when all species were included (R^2 = 0.371, positive slope), when only temperate species were included (R^2 = 0.269, positive slope), and when only tropical species were included (R^2 = 0.304, positive slope). The regression of vessel density against vascular area was statistically significant when all species were included (R^2 = 0.047, negative slope). However, this regression was not significant (Table 2.6) when only temperate species were included (R^2 = 0.0003, positive slope), or when only tropical species were included (R^2 = 0.0003, positive slope).

The regression of vessel diameter distribution skewness against vascular area was not statistically significant (Table 2.6) when all species were included ($R^2 = 0.034$, negative slope), when only temperate species were included ($R^2 = 0.009$, positive slope), or when only tropical species were included ($R^2 = 0.004$, negative slope). The regression of diameter distribution kurtosis against vascular area was statistically significant (Table 2.6) when all species are included ($R^2 = 0.076$, positive slope), and when only temperate species were included ($R^2 = 0.096$, positive slope). This regression was not significant (Table 2.6) when only tropical species were included ($R^2 = 0.038$, positive slope).

The regression of hydraulically weighted diameter of xylem against vascular area was statistically significant (Table 2.6) when all species were included ($R^2 = 0.392$, positive slope), when only temperate species were included ($R^2 = 0.212$, positive slope), and when only tropical species were included ($R^2 = 0.356$, positive slope). The regression of theoretical specific conductivity against vascular area was statistically significant (Table 2.6) when all species were included ($R^2 = 0.232$, positive slope), when only temperate species were included ($R^2 = 0.192$, positive slope), and when only tropical species were included ($R^2 = 0.160$, positive slope). The regression of theoretical specific conductivity in vessel with larger diameter $> 24.00 \mu\text{m}$ against vascular area was statistically significant (Table 2.6) when all species were included ($R^2 = 0.371$, positive slope), when only temperate species are included ($R^2 = 0.262$, positive slope), and when only tropical species are included ($R^2 = 0.384$, positive slope).

2.4 Discussion

2.4.1 *Rhododendron* wood—All wood sampled for *Rhododendron* species was found to be diffuse porous. The proportion of vascular area in stems remained relatively constant at approximately 65% as stem diameter increased in these samples. In general, *Rhododendron* vessel diameters are on the smaller side in comparison to other plants including, temperate trees and shrubs (Nabeshima et al. 2015), desert plants (Medeiros and Pockman 2014), or rain forest trees (Olson et al. 2013). The vessel diameter and its relationship to vascular area in this report are consistent with previous results on other *Rhododendron* species (Noshiro et al. 1995). The vessel diameters found in this study are smaller than most tracheid diameters found for a boreal conifer (Chenlemuge et al. 2015). Because tracheid diameters are characteristically smaller than vessels this result means that *Rhododendron* vessel diameters are particularly small compared to other species with vessels. Narrow vessel diameter is associated with low stem hydraulic conductance (Hacke et al. 2009), and tolerance to freezing (Zanne et al. 2014). Hydraulic conductance of wood depends on vessel diameters, vessel distribution, and vessel density. Plants that have narrow vessels can compensate for this limitation by increasing vessel density, or decreasing the skewness to small diameter sizes. The vessel distributions across these species had a relatively high amount of skewness, Thus, small vessel sizes were not compensated by decreasing skewness. The data in this study showed relatively high vessel density indicating that the effect of narrow vessels was partially compensated by relatively high vessel density.

Vessel density has been found in *Rhododendron* to decrease from thin to thicker stems and from trees to shrubs (Noshiro and Suzuki 1995). Differences in plant

architecture (trees vs. shrubs) did not influence the results because all the accessions were shrubs. Noshiro and Suzuki 1995) found a mean vessel density of 882.9 mm^{-2} for *Rhododendron* shrubs in Nepal, which is similar to the average of all the shrub species used in this study (991.8 mm^{-2}). The stems used for this analysis are in the thinner range of the Noshiro study (Noshiro and Suzuki 1995), which suggests that the density of vessels would be significantly lower if larger stems were used in this study.

The net result of this wood analysis is that *Rhododendron* shrubs are likely to be constrained to low transpiration rates because of the restriction placed on hydraulic flow by the small vessel diameters. The limitation to hydraulic flow characteristic of *Rhododendron* species, should result in relatively low leaf water potentials under conditions of high evaporative demand and open stomata because hydraulic flow would not be able to “keep up with evaporative demand”. Therefore, considering the data set as a whole, *Rhododendron* wood is characteristically “safe” because of narrow vessels and being diffuse porous, which results in low hydraulic conductance, yet *Rhododendron* shrubs ought to be vulnerable to the occurrence of low leaf and stem water potentials because of their wood anatomy.

2.42 Addressing hypothesis one—This hypothesis predicted that the wood of temperate *Rhododendron* species would be significantly safer than the wood of tropical *Rhododendron* species. The results of this research convincingly supported hypothesis one. The mean vessel diameter in temperate species was consistently smaller than that in the tropical species. Also, minimum and maximum vessel diameter in temperate species were significantly smaller than those of tropical species. Vessel density in temperate species tended to be lower than that in the tropical species, but not statistically

significant. These results suggest that temperate *Rhododendron* species do not compensate for their smaller vessel diameter by increasing vessel density to achieve higher water flow. As a consequence, the theoretical specific conductivity was significantly lower in temperate species compared to the tropical species.

The vessel distribution of temperate *Rhododendron* species was skewed toward smaller diameter vessels compared with the less skewed vessel distribution in tropical species. This trend has been found in a dominant desert species that ranges across habitats that have a gradient of air temperature (Medeiros and Pockman 2014). Higher skewness to the smaller size classes means that a disproportionately high proportion of vessels are in small size classes compared to a normal distribution. Also, the study on *Larrea* species by Medeiros and Pockman (2014) suggested that the kurtosis of the vessel distribution was lower in colder habitats because high kurtosis would make the wood vulnerable to a freeze event that induced cavitation in the mean vessel size. Lower kurtosis of vessel distribution insures that there will be vessels smaller than that affected by a particular freeze event. Kurtosis of the temperate species in this study trended lower than that of the tropical species. However, that trend was not statistically significant.

This study on *Rhododendron* species clearly demonstrated that species in temperate habitats have greater vascular safety than species in tropical habitats. The result of that safety is lower potential hydraulic conductance as found with other species (Choat et al. 2011). The low hydraulic conductance in *R. maximum* vascular tissue has been deemed a cause for a characteristically low stomatal conductance (Lipp and Nilsen 1997). Also, *Rhododendron* vascular tissue in temperate regions can be relatively sensitive to drought induced embolism, but can have a high potential to recover from

either drought induced or freeze-thaw induced embolism (Cordero and Nilsen 2002). This research and previous studies by others (Lipp and Nilsen 1997, Cordero and Nilsen 2002) characterized *Rhododendron* wood in temperate regions as having low hydraulic conductance, high vulnerability to drought induced embolism and high safety against freeze-thaw induced embolism. These wood characteristics, along with those from this research, taken together, demonstrate that temperate *Rhododendron* wood ought to restrict *Rhododendron* species to moderate-low light intensity high water availability environments.

2.43 Addressing hypothesis two—Elevation has a large effect on climatic conditions. An increase in elevation causes the air temperature to decrease according to the adiabatic lapse rate. Consequently, dry air decreases temperature by $9.8\text{ }^{\circ}\text{K} / \text{km}$ of elevation change. The rate of temperature decrease with elevation becomes less when air is humidified. Thus, the dry air adiabatic lapse rate is $-0.98\text{ }^{\circ}\text{C} / 100\text{ m}$ increase in elevation while that of humid air is $-0.52\text{ }^{\circ}\text{C} / 100\text{ m}$ (Wikipedia: lapse rate). Thus, species with ranges at high elevation will experience colder conditions than species at low elevation. Moreover, the number of freeze-thaw cycles per winter season increases at higher elevation and at higher latitude (Lipp and Nilsen 1997). The mean elevation of the native range for species used in this study varied from 150 - 4,130 m, which would suggest a decrease in mean air temperature of approximately $20\text{ }^{\circ}\text{C}$ across that range. In a tropical environment, that has a sea level mean temperature of $30\text{ }^{\circ}\text{C}$ would have a mean annual temperature of $10\text{ }^{\circ}\text{C}$ at 4,000 m elevation. In general, the elevation where freeze-thaw events occur is at higher elevation in tropical regions than temperate regions, although there is a lot of variation in the elevation effect on climate because of many

mitigation factors (Körner 2007). Therefore, the strength of the elevation effect on plant traits is likely to be weak because of the variation in mitigating factors affecting the change in habitat conditions among different mountain ranges. Plants have evolved specific functional traits to maximize survivorship at specific elevation ranges. Because wood “safety” is an important adaptation of freeze-thaw events, the wood “safety” is expected to be higher for species with higher native ranges. Therefore, I hypothesized that *Rhododendron* species with a higher elevation of the native range would have safer wood than those species with a lower native range.

To evaluate this hypothesis, three categories of species with low (0 - 1,200 m), medium (1,201 - 2,400 m), and high (2,401 - 4,200 m) native ranges were established to evenly separate species into three categories based on the mean native range reported in the literature. The wood of higher elevation *Rhododendron* species was predicted to be significantly safer than the wood of *Rhododendron* species from lower elevation because the frequency of freezes increases with elevation. Overall, the results weakly support this hypothesis. The mean vessel diameter decreased from the lower to higher elevation rank, but this was not statistically significant. Minimum vessel diameter is significantly different among elevation ranks, but the trend is not consistent. Maximum vessel diameter of species in higher elevation rank is smaller than that of species in the lower ranks, but the difference is non-significant. This trend is inconclusive for vessel density, and not statistically significant. Since the ranked elevation for ANOVA analysis lost some of the information about elevation by making the ranks, a regression analysis was carried out with mean elevation of the native range as the independent variable against the wood traits as dependent variables. There was a significant negative relationship

between mean native elevation and mean vessel diameter or maximum vessel diameter. However, the result of the minimum vessel diameter did not support the hypothesis because there was no significance to the regression. These data suggested that elevation range has a significant impact on mean and maximum vessel diameter, but not the minimum vessel diameter. The range of vessel diameter became smaller at higher elevation.

There was a significant positive regression between vessel density and average native elevation, which suggested that higher elevation plants tend to have denser vessels that might partially compensate for the effect of smaller vessels on stem hydraulic conductance. The vessel distribution skewness is significantly related to native elevation average, and supported the hypothesis that the higher elevation species will have vessel distributions skewed to smaller size classes; however, distribution kurtosis is not significant. One interesting part of the regression analysis was how separation of data into each habitat affected the interpretation. If the analysis included only temperate or only tropical species, the relationship in most of the traits will not be as significant as the analysis as whole. The conclusion is that there is a moderate support for hypothesis two, because some of the vascular traits suggest higher wood safety at high elevation but others do not. Perhaps this weak relationship is a consequence of including species from many different mountains in this analysis. If the wood traits are evaluated among species on one mountain gradient the results may be clearer such as that found earlier (Noshiro and Suzuki 1995).

2.44 Addressing hypothesis three—If microevolutionary processes in temperate habitats have resulted in vascular systems in *Rhododendron* shrubs that constrain water

flow and limit plant growth rate, then these traits would be maladaptive in tropical habitats. Evergreen plants in tropical habitats will encounter greater competition and greater potential damage by herbivores. Therefore, a slow growth rate and relatively long-lived leaves would be maladaptive. There is strong molecular evidence that the main tropical subgenus of *Rhododendron* originated in the temperate zone of southwest China and diversified into tropical habitats (Brown et al. 2006). Thus, I predicted that the wood of tropical *Rhododendron* species would have significantly higher transport efficiency than the wood of temperate *Rhododendron* species. The third hypothesis was strongly supported by the data. The vessel diameters and vessel densities of tropical *Rhododendron* species were found to be significantly larger than those from temperate counterpart, which would enhance potential transportation efficiency in the wood. Moreover, the wood of tropical *Rhododendron* species had significantly higher hydraulically weighted diameter of xylem (D_h) and theoretical specific conductivity (K_s^{theo}) than the temperate species. However, the wood of the tropical species has lost important “safety” parameters because of the larger mean vessel diameters, lower skewness of the vessel distribution and the significantly higher proportion of theoretical transport in vessels diameters $> 24 \mu\text{m}$ ($\Sigma D^4 > 24$) compared with that of temperate species.

2.45 Implications for *Rhododendron* ecology—The results revealed that there is a significant trade-off between safety and efficiency in *Rhododendron* wood. Thus, this research supports the trade-off between safety and the efficiency previously documented in plants (Gleason et al. 2016) even in a narrow phylogenic group. Gleason et al. (2016) point out that it is most advantageous for plants to have high safety and high efficiency,

but this balance has not been attained by plants. Thus, there must be an efficiency cost of high safety (Gleason et al. 2016). The *Rhododendron* species tested fall into the class of high safety and low efficiency, which is at the far right of the trade-off (Gleason et al. 2016). In accordance, temperate *Rhododendron* species have safer wood than their tropical counterparts. The significantly smaller mean, minimum and maximum vessel diameter make temperate *Rhododendron* species less susceptible to freeze-thaw induced embolism. The safe wood provides advantages for *Rhododendron* species in habitats with frequent freeze-thaw events in the winter, but there are consequences of safe wood for hydraulic conductance. The narrow vessel constrains hydraulic conductance and limits the plant to lower transportation rates. Relatively low hydraulic conductance during periods of high evaporative demand will induce low water potential in leaves and cause stomatal closure. Thus, the low hydraulic conductance, characterized by temperate *Rhododendron* wood, will be associated with low photosynthetic rates and a slow growth rate. Moreover, the restriction in theoretical water flow makes the temperate *Rhododendrons* species more vulnerable to low water potential and more likely to experience drought induced embolism. Furthermore, there are very few if any tree form *Rhododendron* species in temperate habitats, most likely due to the low hydraulic conductance of their wood.

As a result of the “safe” wood temperate *Rhododendron* species are restricted to moister habitats with more constant water availability. For example, temperate *Rhododendron* species can grow successfully along stream margins, valleys bottom and on north slopes. In contrast, temperate *Rhododendron* species are uncommon on south facing slopes or ridge tops where the radiation load is higher. The slower growth rate and

restricted distribution range may be associated with poor competitive ability. Yet, there are several *Rhododendron* species that have dominance in understory environments in temperate forests world-wide. For example; *R. maximum* in the eastern deciduous forest of the United States, *R. ponticum* in the deciduous forest of the Black Sea region and *R. brachycarpum* in the birch forest of Japan. These species are the only evergreen species in their respective regions that have strong protection against freezing induced damage in the winter, yet have the ability to root sprout and form dense colonies that inhibit the growth of competitors. Thus, the wood of *Rhododendron* species does constrain their relative growth rate suggesting low competitive ability, yet a suite of other traits along with protection against freeze induced wood damage can result in dominant understory *Rhododendron* shrubs in temperate habitats.

The wood of tropical *Rhododendron* species surveyed in this research is biased toward higher efficiency compared to the temperate species surveyed. The significantly larger vessel size resulted in higher water transport rate, which can enable a higher transpiration rate per leaf area. In addition, higher hydraulic conductance can lead to larger leaf area per wood area (Huber value). The higher transpiration and the ability to have higher stomatal conductance can lead to higher whole plant carbon gain. Consequently, the wood of tropical *Rhododendron* species ought to make these species more competitive than their temperate counterparts in a tropical habitat. Even though the tropical *Rhododendron* wood is biased toward efficiency compared to the wood of temperate *Rhododendron*, it is still relatively safe in comparison to many other tropical plants. This may be one reason why tropical *Rhododendron* species gain dominance at higher elevation in the tropics where protection against freezing becomes important.

One limitation in this study is the bias in plant specimen wood diameters. There was a significant effect of habitat on stem diameter. The temperate *Rhododendron* species sample had smaller mean stem diameter compared to the tropical *Rhododendron* species sampled. Also, larger wood diameter was found to have higher efficiency irrespective of the species habitat. It is possible that the higher efficiency of wood in tropical *Rhododendron* species was biased because of smaller diameter branches in the sampled temperate species. However, the stems were selected from the same age range. Thus, the temperate *Rhododendron* stem is smaller than the tropical *Rhododendron* stem at the same age. Therefore, the bias of stem diameter in this study reflects the reality of stem architecture for species in different habitats and does not detract from the overall conclusion that wood of tropical *Rhododendron* species has higher efficiency than wood of temperate *Rhododendron* species.

2.46 Summary and conclusion—In summary, the data indicate that stems of *Rhododendron* are characteristically “safe” and fall into the high safety and low efficiency end of the trade-off between safety and efficiency. Moreover, there is a significant trade-off between safety and efficiency in *Rhododendron* stems if habitat (temperate vs. tropical) or elevation of the native range is considered. Both evergreen angiosperms in general and the *Ericaceae* alone have been found to have a significant trade-off between safety and efficiency (Gleason et al. 2016). The data add to this general theory by indicating that even within one narrow phylogenetic group that falls at extremes of the trade-off between safety and efficiency, there is still a significant trade-off. Also, this research suggests that the trade-off has important functional significance to the ecology and distribution of *Rhododendron* species.

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2.6 Tables

Table 2.1 List of the *Rhododendron* species used in this study

Species	Habitat	Average Elevation (m)
<i>R. anthopogon</i>	Temperate	3,127
<i>R. augustinii</i>	Temperate	2,350
<i>R. campylogynum</i>	Temperate	3,508
<i>R. catawbiense</i>	Temperate	1,592
<i>R. cinnabarinum</i>	Temperate	3,127
<i>R. dalhousiae</i>	Temperate	2,364
<i>R. edgeworthii</i>	Temperate	2,898
<i>R. forrestii</i>	Temperate	3,737
<i>R. galactinum</i>	Temperate	3,150
<i>R. hippophaeoides</i>	Temperate	3,335
<i>R. huianum</i>	Temperate	2,034
<i>R. hyperythrum</i>	Temperate	1,060
<i>R. hyperythrum</i>	Temperate	1,060
<i>R. keiskei</i>	Temperate	1,220
<i>R. keiskei</i>	Temperate	1,220
<i>R. keysii</i>	Temperate	3,050
<i>R. lepidostylum</i>	Temperate	3,355
<i>R. lindleyi</i>	Temperate	2,745
<i>R. macrophyllum</i>	Temperate	600
<i>R. megeratum</i>	Temperate	3,830
<i>R. minus</i>	Temperate	1,700
<i>R. minus</i> var. <i>Chapmanii</i>	Temperate	150
<i>R. orbiculare</i>	Temperate	3,150

<i>R. ponticum</i>	Temperate	900
<i>R. pronum</i>	Temperate	4,130
<i>R. proteoides</i>	Temperate	4,118
<i>R. pseudochrysanthum</i>	Temperate	2,898
<i>R. racemosum</i>	Temperate	3,050
<i>R. ririei</i>	Temperate	1700
<i>R. rubiginosum</i>	Temperate	3,355
<i>R. sherriffii</i>	Temperate	3,661
<i>R. spinuliferum</i>	Temperate	2,200
<i>R. strigillosum</i>	Temperate	2,828
<i>R. tephropeplum</i>	Temperate	3,370
<i>R. trichostomum</i>	Temperate	3,400
<i>R. walongense</i>	Temperate	1,830
<i>R. williamsianum</i>	Temperate	2,800
<i>R. aurigeranum</i>	Tropical	1,328
<i>R. bryophilum</i>	Tropical	1,448
<i>R. celebicum</i>	Tropical	1,900
<i>R. crassifolium</i>	Tropical	2,135
<i>R. densifolium</i>	Tropical	1,400
<i>R. himantodes</i>	Tropical	1,650
<i>R. jasminiflorum</i>	Tropical	1,300
<i>R. javanicum</i>	Tropical	1,508
<i>R. kawakamii</i>	Tropical	1,900
<i>R. kochii</i>	Tropical	1,200
<i>R. laetum</i>	Tropical	1,968
<i>R. multicolor</i>	Tropical	1,500
<i>R. orbiculatum</i>	Tropical	1,300

<i>R. radians</i>	Tropical	1,600
<i>R. rarilepidotum</i>	Tropical	2,235
<i>R. rushforthii</i>	Tropical	1,650
<i>R. rushforthii</i>	Tropical	1,650
<i>R. suaveolens</i>	Tropical	1,500
<i>R. taxifolium</i>	Tropical	2,650
<i>R. tuba</i>	Tropical	2,520
<i>R. verticillatum</i>	Tropical	1,100
<i>R. yongii</i>	Tropical	2,490
<i>R. zoelleri</i>	Tropical	675

Table 2.2 Two-way ANOVA table for the effects of habitat, elevation rank, and their interaction on vessel traits of *Rhododendron* species. Mean vessel = mean vessel diameter (μm); Min vessel = minimum vessel diameter (μm); Max vessel = maximum vessel diameter (μm); Vessel density = the number of vessels per wood area sampled (mm^{-2}).

Vessel Traits	DF	SS	F	Sig.
Mean Vessel				
Habitat	1	101.06	15.20	0.0003***
Elevation Rank	2	24.25	1.82	0.17
Habitat x Elevation	2	16.29	1.22	0.30
Min Vessel				
Habitat	1	13.45	4.45	0.0394*
Elevation Rank	2	19.07	3.16	0.05*
Habitat x Elevation	2	14.38	2.38	0.10
Max Vessel				
Habitat	1	131.36	6.65	0.0127*
Elevation Rank	2	49.09	1.24	0.30
Habitat x Elevation	2	49.85	1.26	0.29
Vessel Density (pV)				
Habitat	1	102603.66	2.20	0.14
Elevation Rank	2	63939.97	0.69	0.51
Habitat x Elevation	2	21159.49	0.23	0.80

Table 2.3 Two-way ANOVA table for the effects of habitat, elevation rank, and their interaction on the skewness and kurtosis of vessel diameter distributions in

Rhododendron species stem.

Vessel Distribution	DF	SS	F	Sig.
Skewness				
Habitat	1	0.36	7.21	0.0096***
Elevation Rank	2	0.45	4.59	0.0145**
Habitat x Elevation	2	0.24	2.41	0.10
Kurtosis				
Habitat	1	0.04	0.11	0.75
Elevation Rank	2	0.04	0.05	0.95
Habitat x Elevation	2	0.61	0.87	0.42

Table 2.4 Two-way ANOVA table for the effects of habitat, elevation rank, and their interaction on the wood transport efficiency. D_h = Hydraulically weighted diameter of xylem, K_s^{theo} = theoretical specific conductivity, $\Sigma D^4 > 24$ = the proportional of the theoretic transport in vessels that occurs in vessels greater than 24 μm in diameter.

Wood Transport Efficiency	DF	SS	F	Sig.
D_h				
Habitat	1	111.32	14.24	0.0004***
Elevation Rank	2	28.88	1.85	0.17
Habitat x Elevation	2	19.06	1.22	0.30
K_s^{theo}				
Habitat	1	6.84	6.62	0.0131***
Elevation Rank	2	2.25	1.09	0.34
Habitat x Elevation	2	7.32	3.54	0.0363*
$\Sigma D^4 > 24$				
Habitat	1	1752.65	11.25	0.0015*
Elevation Rank	2	1073.50	3.45	0.0391*
Habitat x Elevation	2	501.57	1.61	0.21

Table 2.5 *P*-values for regressions between wood traits (as dependent variable) and mean elevation of the native range (as independent variable), including all species (All data), only temperate species (Temperate), and only tropical species (Tropical). Mean vessel = mean vessel diameter (μm); Min. vessel = minimum vessel diameter (μm); Max. vessel = maximum vessel diameter (μm); Vessel density = the number of vessels per wood area sampled. D_h = Hydraulically weighted diameter of xylem (μm), K_s^{theo} = theoretical specific conductivity ($\text{kg m}^{-1} \text{MPa}^{-1} \text{s}^{-1}$), $\Sigma D^4 > 24$ = the proportional of the theoretic transport in vessels that occurs in vessels greater than 24 μm in diameter (%).

Traits	All Data	Temperate	Tropical
Mean Vessel	0.0028***	0.0919*	0.41
Min. Vessel	0.37	0.42	0.078*
Max. Vessel	0.0012***	0.0097***	0.82
Vessel Density	0.0533*	0.57	0.18
Skewness	0.0037***	0.53	0.0054***
Kurtosis	0.17	0.11	0.39
D_h	0.0016***	0.057*	0.36
K_s^{theo}	0.0465	0.0752*	0.38
$\Sigma D^4 > 24$	0.0029***	0.0165**	0.0572*

Table 2.6 Results of regressions between wood traits (as dependent variable) and vascular area (as independent variable), including all species (All data), only temperate species (Temperate), and only tropical species (Tropical). Mean vessel = mean vessel diameter (μm); Min. vessel = minimum vessel diameter (μm); Max. vessel = maximum vessel diameter (μm); Vessel density = the number of vessels per wood area sampled. D_h = Hydraulically weighted diameter of xylem (μm), K_s^{theo} = theoretical specific conductivity ($\text{kg m}^{-1} \text{MPa}^{-1} \text{s}^{-1}$), $\Sigma D^4 > 24$ = the proportional of the theoretic transport in vessels that occurs in vessels greater than 24 μm in diameter (%).

Traits	All Data	Temperate	Tropical
Mean Vessel	<0.0001***	0.0083***	0.0038***
Min. Vessel	0.21	0.41	0.55
Max. Vessel	<0.0001***	0.001***	0.0063***
Vessel Density	0.0957*	0.91	0.30
Skewness	0.15	0.56	0.76
Kurtosis	0.0324**	0.0607*	0.37
D_h	<0.0001***	0.0041***	0.0026***
K_s^{theo}	0.0001***	0.0067***	0.0803**
$\Sigma D^4 > 24$	<0.0001***	0.0012***	0.0016***

2.7 Figure legends

Figure 2.1 A cross section micrograph at 40 x objective lens of *R. jasminiflorum* stem.

Vessel element (A), fiber (B), and ray cell (C) are shown.

Figure 2.2 (A) Box plots of mean vessel diameter among all species, separated by temperate and tropical habitats, and separated by elevation ranks, (B) Box plots of minimal vessel diameter among all species, separated by temperate and tropical habitats, and separated elevation ranks, (C) Box plots of maximum vessel diameter among all species, separated by temperate and tropical habitats, and separated by elevation ranks, (D) Box plots of mean vessel density among all species, separated by temperate and tropical habitats, and separated by elevation ranks. Elevation ranks are low (0 - 1,200 m), medium (1,201 - 2,400 m), and high (2,401 - 4,200 m).

Figure 2.3 (A) Box plots of mean skewness of the vessel diameter distribution among all species, separated by temperate and tropical habitats, and separated by elevation ranks, (B) Box plots of mean kurtosis of the vessel diameter distribution among all species, separated by temperate and tropical habitats, and separated by elevation ranks. Skewness and kurtosis were determined using JMP Pro 12. Elevation ranks are low low (0 - 1,200 m), medium (1,201 - 2,400 m), and high (2,401 - 4,200 m).

Figure 2.4 (A) Box plots of the hydraulically weighted diameter of xylem (D_h) among all species, separated by temperate and tropical habitats, and separated by elevation ranks, (B) Box plots of the mean theoretical specific conductivity (K_s^{Theo}) among

all species, separated by temperate and tropical habitats, and separated by elevation ranks, (C) Box plots of the mean and standard deviation of the proportion of theoretical transport in vessel with larger diameter ($> 24.00 \mu\text{m}$) ($\Sigma D^4 > 24$) among all species, separated by temperate and tropical habitats, and separated by elevation ranks. Elevation ranks are low (0 - 1,200 m), medium (1,201 - 2,400 m), and high (2,401 - 4,200 m).

Figure 2.5 (A) Regression of vessel diameter (as dependent variable) against vessel density (as independent variable) including all species ($R^2 = 0.059$), (B) for temperate species only ($R^2 = 0.011$), (C) for tropical species only ($R^2 = 0.017$).

Figure 2.6 (A) Regression of wood stem cross section area (as dependent variable) against whole stem area (as independent variable) including all species ($R^2 = 0.951$), (B) for temperate species only ($R^2 = 0.937$), (C) for tropical species only ($R^2 = 0.944$).

2.8 Figures

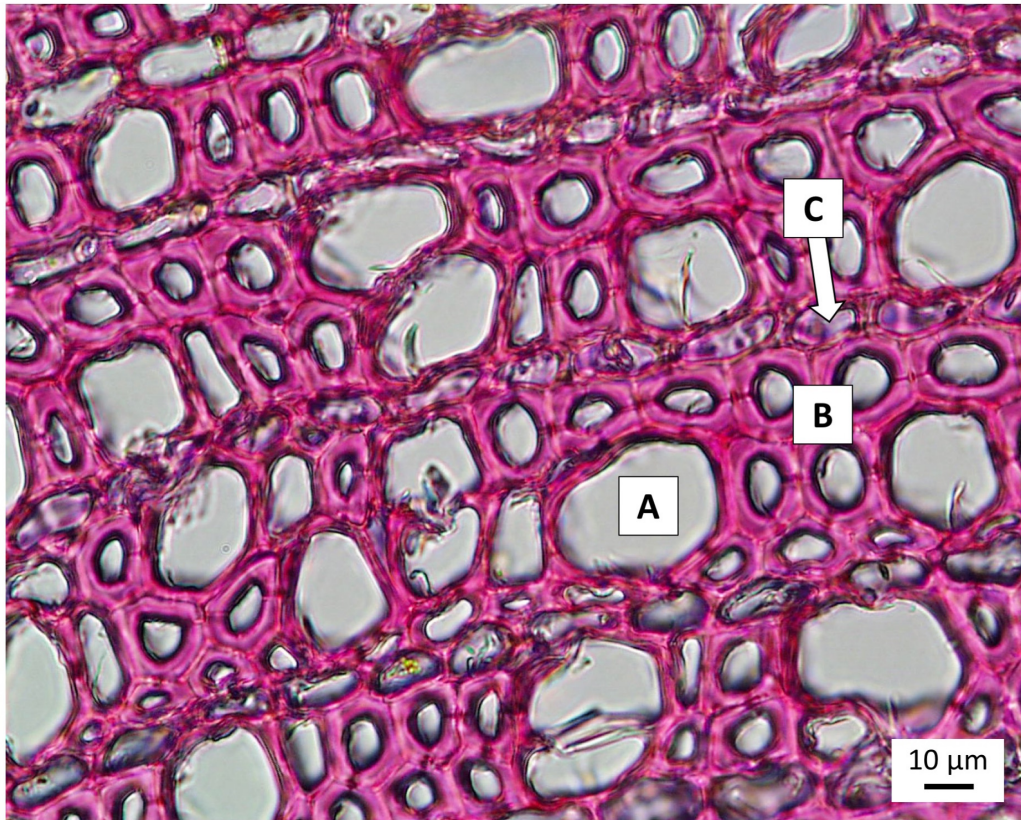


Figure 2.1. A cross section micrograph of *R. jasminiflorum* stem

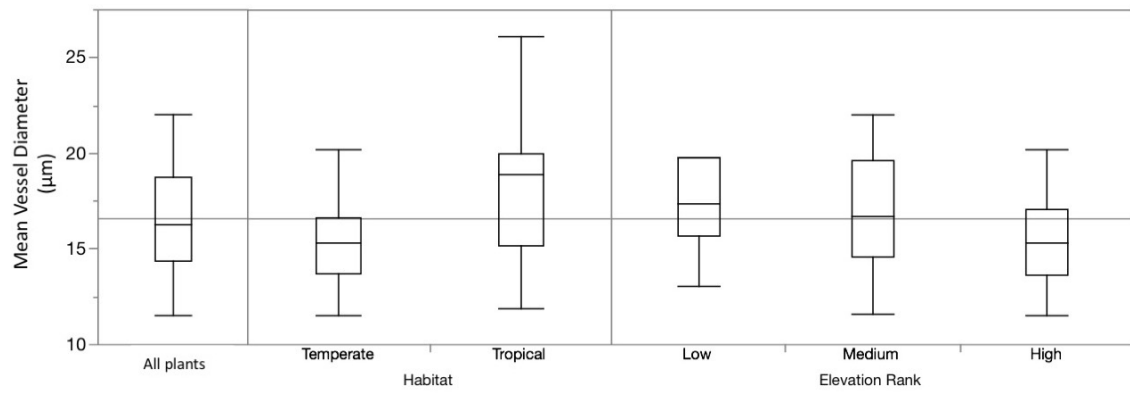


Figure 2.2 A

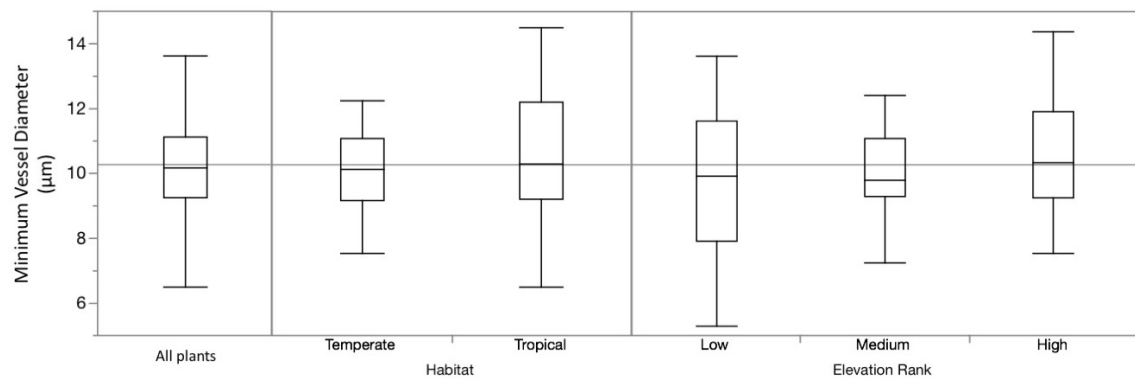


Figure 2.2 B

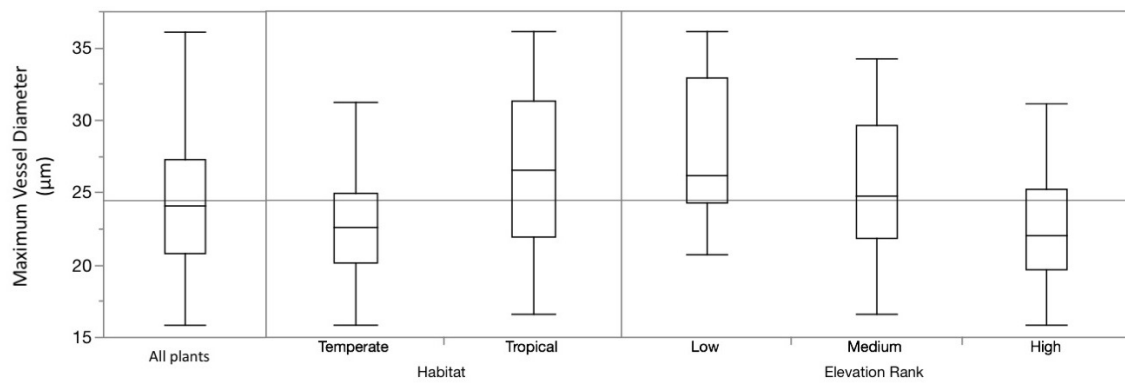


Figure 2.2 C

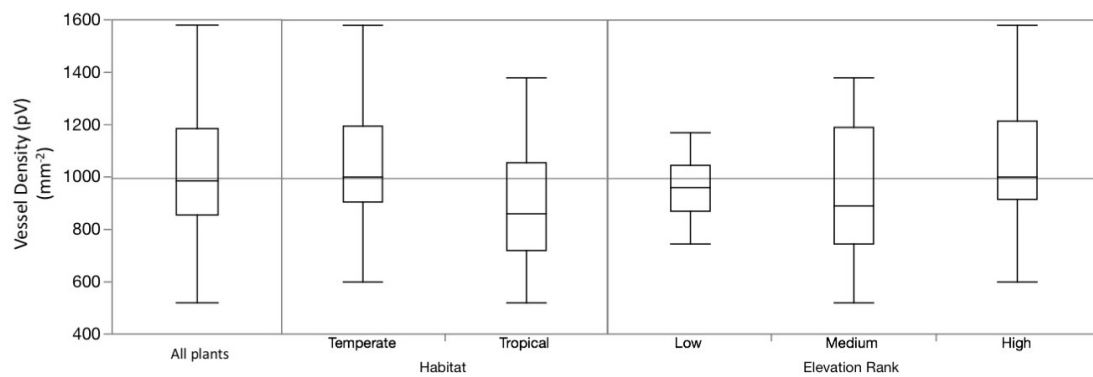


Figure 2.2 D

Figure 2.2 Box plots of vessel traits

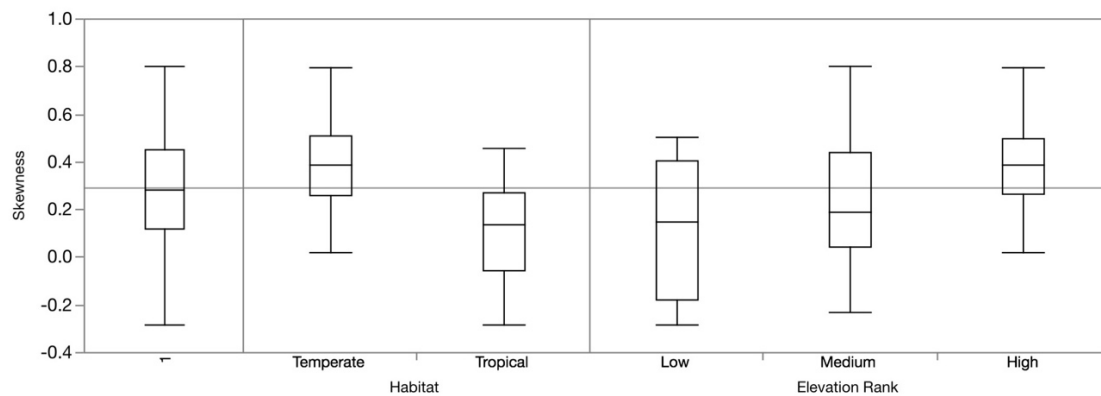


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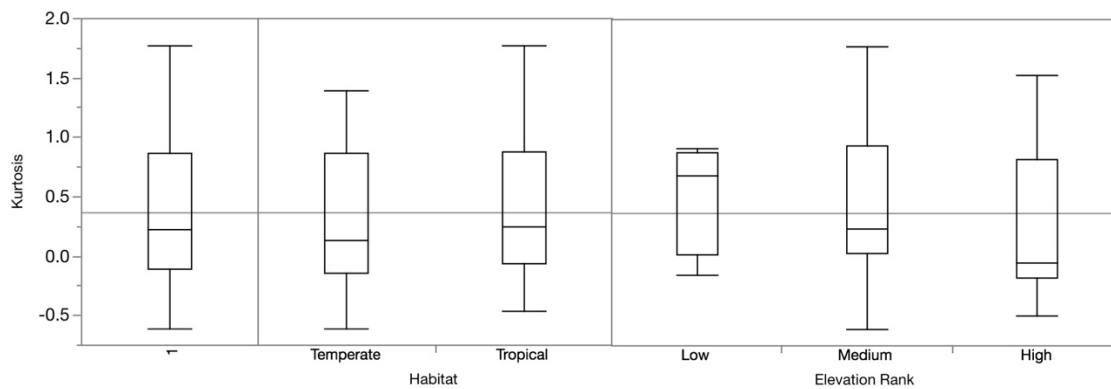


Figure 2.3 B

Figure 2.3. Box plots of mean skewness and kurtosis

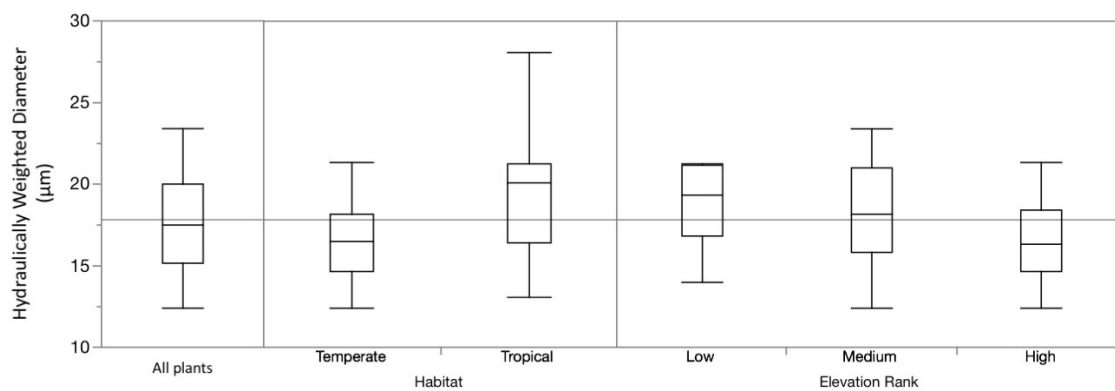


Figure 2.4 A

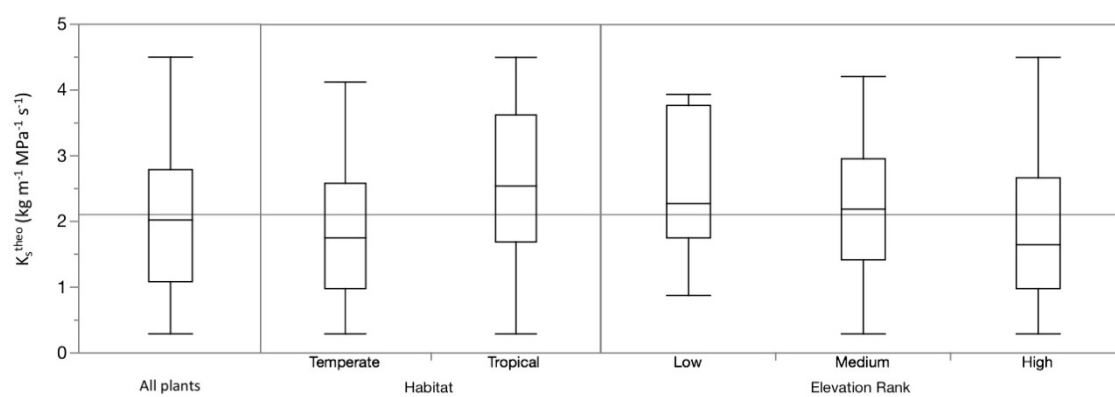


Figure 2.4 B

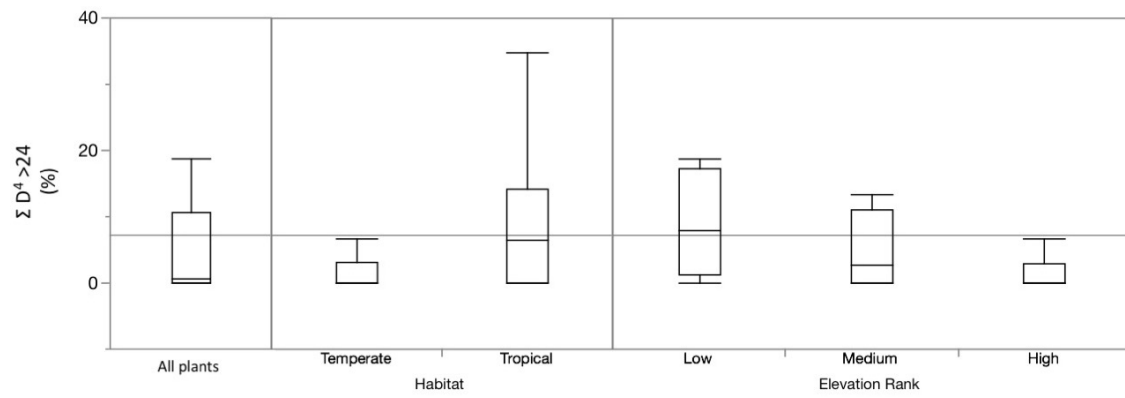


Figure 2.4 C

Figure 2.4. Box plots of the hydraulic traits

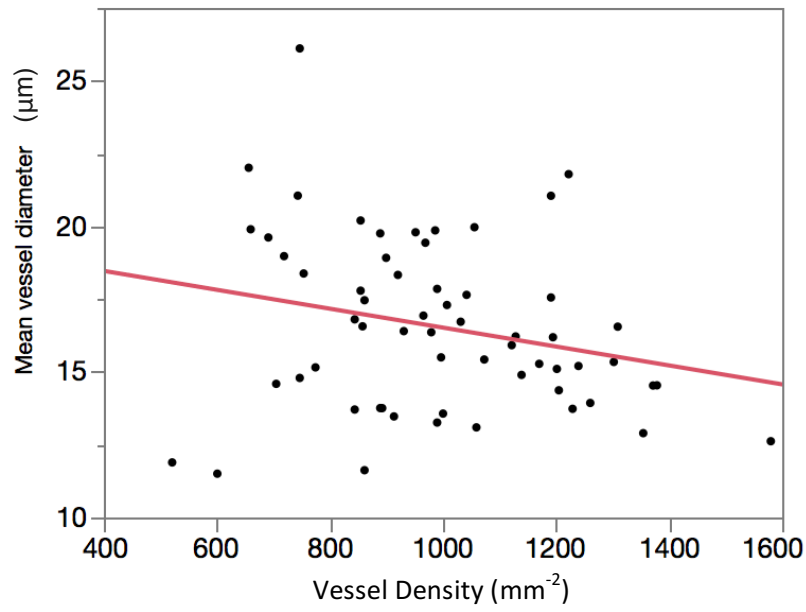


Figure 2.5 A

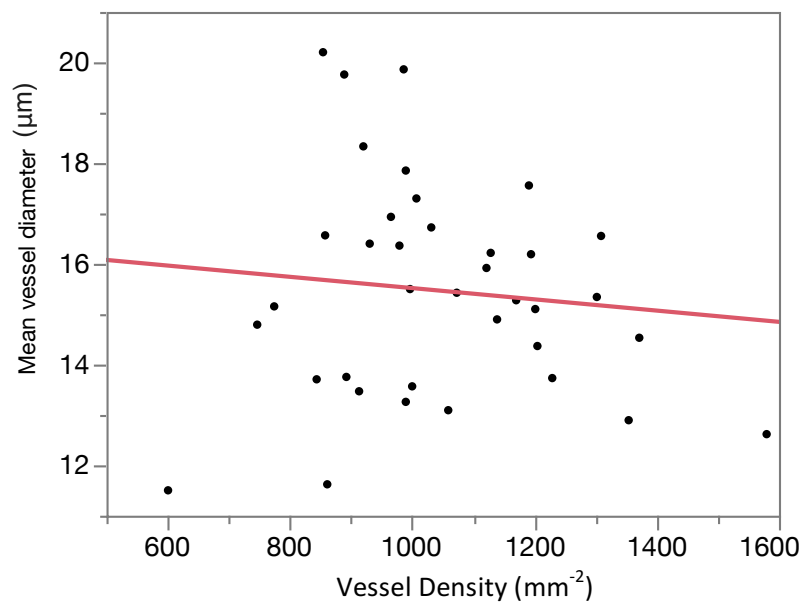


Figure 2.5 B

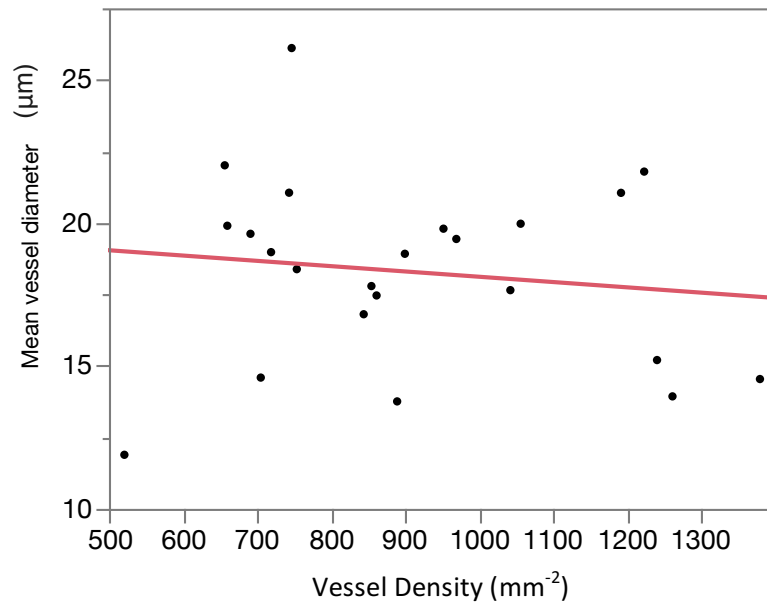


Figure 2.5 C

Figure 2.5. Regression of vessel diameter against vessel density

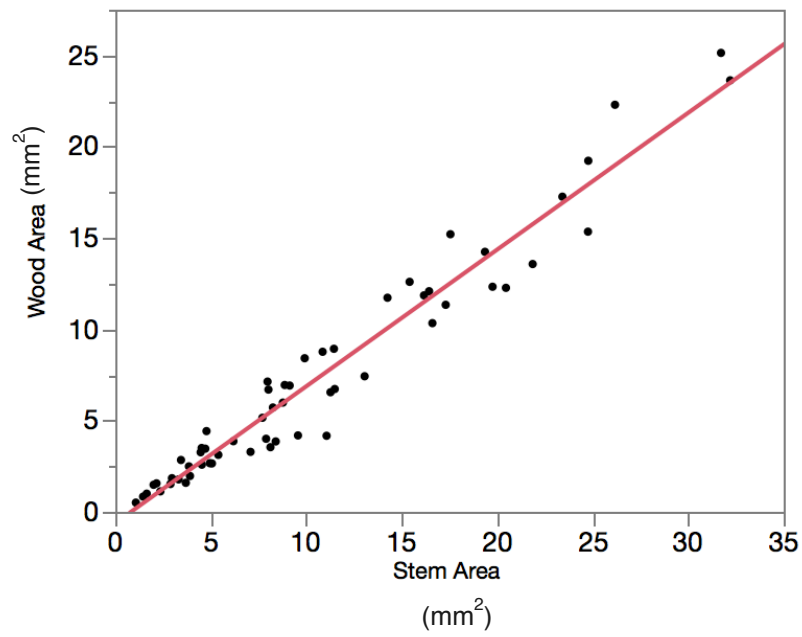


Figure 2.6 A

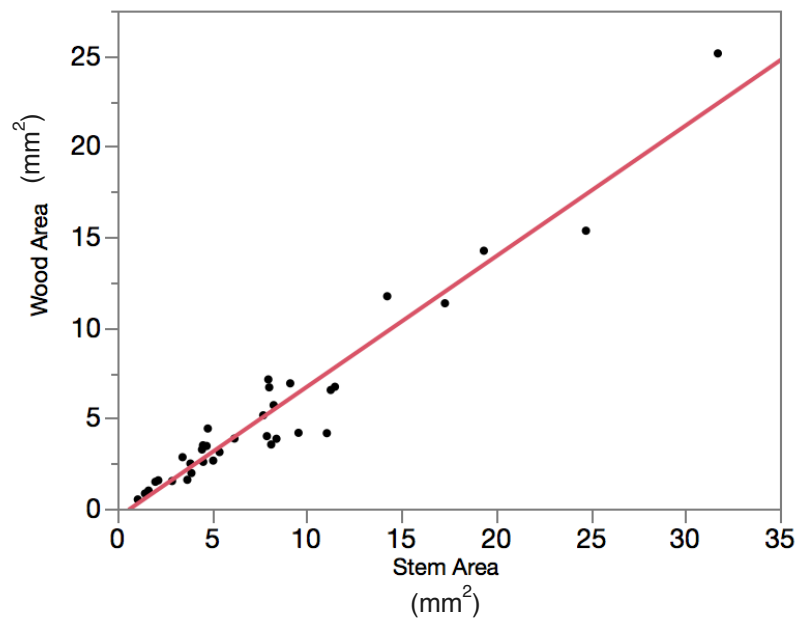


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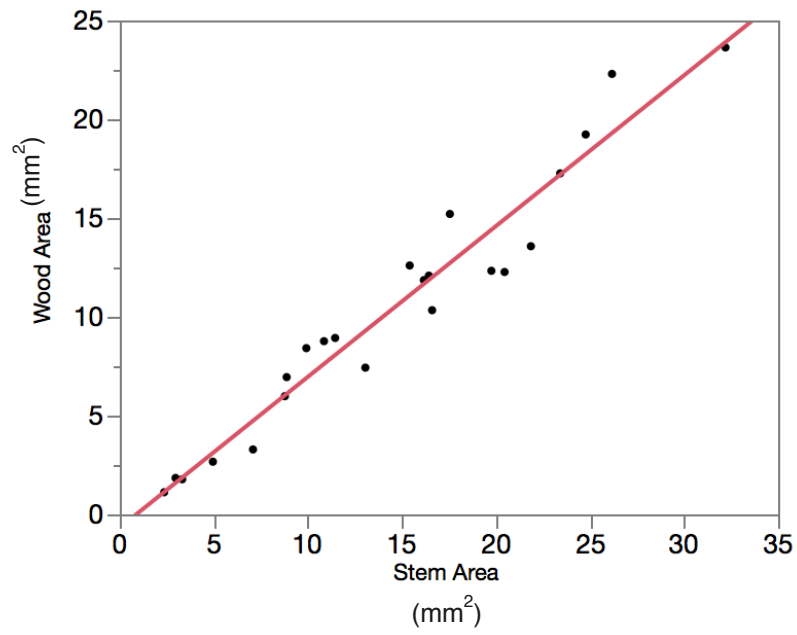


Figure 2.6 C

Figure 2.6. Regression of wood area against whole stem area

CHAPTER 3

Exploring leaf anatomical traits of *Rhododendron* species: A focus on the relationship with elevation and habitat (temperate vs. tropical) of the native range.

3.1 Introduction

It is well known that leaf anatomical traits have important implications to plant fitness through their effects on carbon gain by photosynthesis (Brodribb and Feild 2010). In particular, the total amount of leaf area per ground area (leaf area index) is highly related to plant productivity (Ewert 2004). Also, plant growth rate is significantly related to specific leaf weight (SLW) such that species with relatively thin leaves (low SLW) usually have a faster growth rate than plants with relatively high SLW (Chapin 1980). In general, leaves with higher SLW (usually thicker leaves) have a higher carbon cost per leaf area, but have a lower nitrogen content per leaf area, which results in a low photosynthetic rate per leaf area and slower whole plant growth rate (Chapin 1980). Also, plant productivity is related to the capacity for water flow from roots to leaves. The amount of water flow per vascular area per leaf area is termed leaf hydraulic conductance. Plants with high leaf hydraulic conductance have a higher plant productivity than plants with low leaf hydraulic conductance (Tyree 2003). Because leaf hydraulic conductance is directly related to stomatal pore index and vein density these leaf functional traits relate directly to plant productivity (Sack et al. 2003). Thus, there are several leaf morphological and anatomical traits that have been identified as functional traits that have significant effects on plant growth rate, competitive ability and distribution on the landscape (Price et al. 2014).

The Leaf Economic Spectrum (LES) defines the relationships between key leaf functional traits and plant resource use efficiency or growth rate (Reich 2014). For instance, leaf specific weight is linearly related to leaf longevity, and leaf longevity is linearly related to whole plant growth rate when plants from across the globe including all biomes are included. Thus, the relationships in the LES define universal fundamental characteristics across plants in all ecosystems. One important determinant of the LES is the number of veins per leaf area VLA (Sack et al. 2013), which is directly correlated with stomatal pore index (SPI) and leaf hydraulic conductance (LHC). Thus, there are positive significant relationships between SPI or LHC and plant growth rate when a global view of plants is considered. One limitation of the LES is that the relationships are presented as log-log plots, which hides some of the variation across the gradient. In fact, the variation within one end of the gradient may be very important to the ecology of that group. For example, at one end of the LES gradient are plants that have relatively thick long-lived leaves, with a low SPI and low LHC that result in a slow growth rate and high nutrient use efficiency. However, leaf epidermal structures such as scales or trichomes can greatly influence leaf function for this group (Brodribb and Hill 1997, Nilsen et al. 2014). Therefore, variation in leaf anatomy or morphology within any functional group may have important implications to plant success. Understanding the variation of anatomical traits within a functional group and its association with habitat conditions can yield significant insight to the ecological adaptations of the group.

There are several reasons why species of evergreen *Rhododendron* constitute a valuable resource for understanding the effects of variation in anatomical traits within a functional group on plant functional ecology. The genus *Rhododendron* is a strongly

monophyletic group in the Ericaceae (Kron and Judd 1990b). The group evolved in the circumboreal forest of Laurasia (Milne 2004). In fact, the vast majority of evergreen *Rhododendron* species have the same general growth habit. They have entire leaves and a woody growth form (Cox 1990). Shoots elongate rapidly and produce leaves in a whorl near the apex of the stem (Nilsen et al. 1987, Nilsen et al. 2014). Although there is high consistency in general growth characteristics, there is considerable variation in overall plant architecture and habitats occupied by *Rhododendron* species. For example, the native ranges of *Rhododendron* species include alpine, arctic, temperate and tropical regions. Moreover, elevation of the native range varies from sea level to 5,000 m. Plants have a wide range in leaf size ($0.5 \text{ cm}^2 - 3,000 \text{ cm}^2$), a diversity of abaxial epidermal appendages (trichomes), and a wide range in plant height from cushion plant to canopy tree. Thus, evergreen *Rhododendron* species reside in one specific location on the LES (high SLW, long lived leaves, low nitrogen concentration, slow growth rate) yet they have significant variation in native ranges and plant architecture that should select for variation in leaf functional traits.

The overall objective of this research was to understand the relationships of habitat and elevation with leaf anatomical traits in a selection of evergreen *Rhododendron* species. Species from tropical and temperate habitats included in this study range from sea level to 4,200 m elevation. Leaves of tropical species, particularly in wet tropical regions, are characterized as hygromorphic (Roth 2012). In table 5 on page 422 of Roth (2012), hygromorphic leaves tend to have the following characteristics: thin lamina, thin cuticle, thin mesophyll relative to leaf thickness, single epidermis, no water storage cells below the epidermis, open mesophyll, low stomatal density, large stomata, no stomatal

crypts and no hairs (trichomes). Based on the fact that tropical *Rhododendron* species in tropical habitats should have more hygromorphic leaves than that of species in temperate habitats the following hypotheses were posed:

1) Leaves on *Rhododendron* species that inhabit tropical regions have functional traits that reflect higher hygromorphy than that of temperate *Rhododendron* species. This hypothesis would be supported if: (a) Leaves on tropical *Rhododendron* species are larger and thinner than that of temperate species. (b) Leaves on tropical species have lower stomatal density and larger stomata than that of temperate species. (c) Leaves of tropical *Rhododendron* species do not have a hypodermis or water storage cells below the epidermis.

2) The leaves of *Rhododendron* species trend toward greater xeromorphy as the native range of those species increases in elevation. This hypothesis would be supported if: (a) Leaf area decreases as the elevation of the native range increases. (b) The stomatal pore index decreases as elevation of the native range increases. (c) There is a higher likelihood that leaves of plants with a native range at higher elevation have hairs or stomata in crypts. (d) The incidence of water storage cells increases as the elevation of the native range increases.

3) The leaf stomatal pore index will increase in accordance to an increase in stem theoretical specific conductivity. This hypothesis would be supported if: (a) The stomatal pore index (dependent variable) measured in this chapter has a significant positive regression with the theoretical specific conductivity (independent variable) of stems measured in chapter 2 (only for common species sampled in chapter 2 and 3).

3.2 Materials and Methods

3.21 Selected accessions—More than 80 accessions (one accession per species) were selected for this research (Table 3.1). Evergreen shrubs that have either a temperate (41 out of 86 species) or tropical (45 out of 86 species) range were selected. This selection effort was used to control overall growth habit and exclude external variables for temperate and tropical regions species. Leaves of species were collected from various sources including the Rhododendron Species Foundation in Federal Way, WA, a common garden in Volcano Village, HI, the Royal Botanic Garden Edinburgh, UK, and Biological Sciences-VBI Plant Growth Facility (VT-PGF).

3.22 Leaf traits surveyed—Four recently mature leaves from outer canopy in four directions were collected from each accession. The leaves were sealed in zip-lock plastic bags and transported to the lab for morphological and anatomical analyses. The leaf length, width and area were measured for each leaf. Leaf length divided by leaf width was used as an index of leaf lamina shape (high value = linear, low value = ovoid). Leaf length times leaf width was used to estimate leaf area from a previously published regression (Nilsen et al. 2014). A digital image (brand and model of the camera) of the abaxial leaf surface was recorded at 10 x through stereo microscope (model SZ-ST; Olympus Optical Co. Ltd., Tokyo, Japan) in order to determine the type of abaxial leaf surface appendages (trichomes). The abaxial surfaces were defined into the following categories modified from Argent (2015), and Nilsen et al. (2014): (1) No trichomes present, (2) Peltate scales (flat wide cap with a short base) present, (3) Cushion scales (inflated wide cap with short base) present, (4) Cuneate scales (cup shaped cap with irregular margin and short base) present, (5) Stellate scales (cup shaped cap with long

finger like projections and a short base) present, (6) Dendroid trichomes (stellate cap on a long stalk subtended by a mound of epidermal cells) present, (7) Hair trichomes (long single cell extension of the epidermis) present, (8) Multi-branched trichomes (long and branched extension of the epidermis composed of multiple cells leading to a mat of tangled hairs) present.

The abaxial surface of three leaves were randomly selected for stomatal imprint (Hilu and Randall 1984). Leaves were painted (between two main lateral veins) with clear nail polish (product # 504; Clairol Inc., Stamford, Connecticut). When the polish was dry and hardened, a narrow piece of clear adhesive tape (Scotch brand package sealing tape) was placed over the nail polish, pressed down firmly, carefully lifted to remove the dried sheet of nail polish (the peel), placed (with the peel facing upward) on a microscope slide and covered with a cover-slip. Five randomly located images (25 x objective lens) of the epidermal peels were captured with a digital camera (model DP-10; Olympus Optical Co. Ltd., Tokyo, Japan) mounted on a bright field microscope (model BX50; Olympus Optical Co. Ltd., Tokyo Japan). All stomata were counted in the field of view of each image to determine stomatal density. Five additional images (40 x objective lens) were captured from the same epidermal peel for stomatal size. The length of every stomatal pore and the total number of pores in the field of view were determined using ImageJ software (National Institutes of Health (NIH), Bethesda, MD). The stomata pore index (SPI) was calculated for each image as: $D (L^2)$. Where D = stomata density (mm^{-2}), and L = mean stomatal pore length (μm) (Parlange and Waggoner 1970).

Following the removal of nail polish, five sections (each 3 - 5 mm in width) from the mid-vein to the margin were excised from each leaf (avoiding the nail polish affected

area) and preserved in FAA (50% ethanol, 35% water, 10% formalin, 5% glacial acetic acid). Following complete preservation (approximately 1 week), sections were sequentially hydrated to remove the FAA, dehydrated in an alcohol gradient (5 steps at 30 minutes each), saturated with xylene (two 1 hr soaks), impregnated with paraffin oil (1 step with 50% paraffin oil and xylene for 1 hr plus 2 steps of 1 hr each of pure paraffin oil), and equilibrated in hot paraffin (3 steps of 8 hr each). The leaf pieces were trimmed and mounted in paraffin for laminal cross sections (8 μm) made with a rotary microtome (model HN 340E; Microm International GmbH, Walldorf, Germany). One ribbon (8 - 10 cross sections) was mounted from one of the leaf pieces from each leaf. The resulting tissue sections were double stained with safranin O and fast green before permanent mounting with Permounttm.

Three to five digital images were taken at (10 x objective lens) from the best sections on each slide (Figure 3.1). The leaf thickness, mesophyll thickness, and number of adaxial epidermal layers were measured using ImageJ software. Some leaves had idioblasts on the adaxial surface under or in the epidermis. The length and width of each idioblast in the image was measured. In leaves that had idioblasts present, the density of idioblasts per mm of leaf length ($ID = \# \text{ idioblast} / \text{length of the section}$) was calculated. Also, the area of each idioblast was calculated by using the geometric formula for the area of an ellipse ($IA = \pi ab$), where a = length and b = width (Selby 1972). The IAs were summed and divided by the total area of the section to attain an index of idioblast expression ($IE = ((\sum IA) / \text{total section area}) \times 100$). In addition, the area of the mesophyll in relation to the area of the leaf (% mesophyll) was determined on each photomicrograph and averaged for each accession. Also, five replicate lines were placed across the leaf

cross-section images. The number of cell walls crossed by each line in the interior of the leaf divided by two is equal the A_{mes}/A ratio. This value represents the density of mesophyll cells and reflects the extent of open space in the spongy mesophyll. High value of A_{mes}/A ratio represent denser spongy mesophyll.

3.23 Statistical analysis—Accessions were assigned to a habitat (temperate or tropical) based on their known native range reported in the literature. Tropical species were those that had ranges between 20 °N and 20 °S latitude. Temperate species had ranges between 30 °N and 40 °N latitude. An elevation was assigned to each species as the middle elevation of the native range reported in the literature. In order to convert these elevation data from continuous to categorical three categories were defined (low = 0 - 1,200 m, medium = 1,201 - 2,400 m, and high = 2,401 - 4,200 m). Two-way ANOVA was used to test the effects of habitat (temperate vs. tropical) and elevation (low, medium, and high) on all morphological and anatomical traits (JMP Pro 12, SAS Institute Inc., Cary, NC). One-way ANOVA was used to test for the effect of elevation on idioblast traits, because idioblast was only found in leaves of tropical species in this study. Spearman correlation and linear regression analyses were used to determine the relationship between stomatal pore index (SPI) and theoretical specific conductivity (K_s^{theo}) in stems of the same species (data from chapter 2).

3.3 Results

3.31 Leaf morphology and anatomy—*Rhododendron* leaves have big variation in size yet the shape, defined by the length to width ratio, is relatively constant. One of the most obvious aspects of *Rhododendron* leaf anatomy was the presence of idioblasts.

Generally, idioblasts were found underneath the adaxial epidermis (Figure 3.1 A [A]), but in some species, idioblasts can be found underneath abaxial epidermis as well (Figure 3.1 A [C]). Palisade mesophyll was found to vary from one to four layers (Figure 3.1 A [B, D, and F]). Each layer of palisade mesophyll was very uniform in cell shape and size, even when disturbed by the presence of idioblasts. Some temperate plants had a hypodermis, a layer of square cells below the epidermis, which varied from one to three layers (Figure 3.1 A [F]). No hypodermis was found in any tropical plants included in this research. Abaxial papillae, short finger like extensions of epidermal cells, can be found in some temperate plants (Figure 3.1 A [F]). *Rhododendron* species have a wide variety of different leaf trichome types (Figure 3.1 B). They were seven different types found among the sampled species. Stomata shape did not vary much, and most stomata were randomly distributed on the abaxial surface. Figure 3.1 C [A] shows a uniform distribution of stomata among papillae surface compare to smooth surface of Figure 3.1 C [B]. Stomata were located in stomatal crypts for a few species particularly *R. himantodes*. All plants were hypostomatous.

The mean and standard deviation of leaf area (Figure 3.2 A) of all data was $16.98 \pm 14.91 \text{ mm}^2$. The mean leaf area of tropical plants ($18.46 \pm 15.45 \text{ mm}^2$) was not significantly different (Table 3.2) from the temperate species ($15.40 \pm 14.33 \text{ mm}^2$). The mean leaf area decreased (Figure 3.2 A) from low elevation to high elevation ($30.15 \pm 17.09 \text{ mm}^2$, $16.97 \pm 13.02 \text{ mm}^2$, and $12.71 \pm 13.85 \text{ mm}^2$ respectively) and elevation effect was statistically significant (Table 3.2). The interaction between habitat and elevation was not significant (Table 3.2). The mean and standard deviation of leaf length to width (L/W) ratio (Figure 3.2 B) of all data was 2.88 ± 2.50 . The mean leaf L/W ratio of

tropical plants (3.18 ± 3.39) was not significantly different (Table 3.2) from the temperate species (2.56 ± 0.71). The mean leaf L/W ratio was from low elevation to high elevation were 2.49 ± 0.60 , 3.11 ± 3.08 , and 2.78 ± 2.23 respectively). The elevation and the interaction between habitat and elevation were not significant (Table 3.2).

The mean and standard deviation of leaf thickness (Figure 3.2 C) of all data was 0.40 ± 0.14 mm. The tropical plants had a significantly (Table 3.2) thicker leaf (0.46 ± 0.13 mm²) than that of the temperate plants (0.31 ± 0.010 mm). The mean leaf thickness (Figure 3.2 C) from low to high elevation were 0.36 ± 0.08 , 0.43 ± 0.15 , and 0.35 ± 0.14 mm respectively. The effect of elevation was not significant (Table 3.2). The interaction between habitat and elevation rank was not significant (Table 3.2). The mean and standard deviation of mesophyll percentage (Figure 3.2 D) of all data was $68.29.78 \pm 10.82$ %. The mean mesophyll percentage of temperate and tropical plants was 68.55 ± 7.16 , and 68.10 ± 12.84 % respectively, but the effect of habitat was not significant (Table 3.2). There was an increasing trend in leaf mesophyll percentage from low ($64.74 \pm 9.76\%$) to medium ($68.49 \pm 12.15\%$) to high ($69.87 \pm 8.79\%$), but the elevation effect was not significant (Table 3.2). However, the interaction between habitat and elevation was statistically significant (Table 3.2). The mean and standard deviation of A_{mes}/A ratio (Figure 3.2 E) of all data is 9.45 ± 2.70 . The mean A_{mes}/A ratio of tropical plants (9.90 ± 2.50) was significantly higher (Table 3.2) than that of the temperate species (8.83 ± 2.89). The mean A_{mes}/A ratio increased from low elevation to high elevation (8.75 ± 3.38 , 9.56 ± 2.54 , and 9.61 ± 2.68 respectively), but the effect of elevation was not significant (Table 3.2). However, the interaction between habitat and elevation was significant (Table 3.2).

3.32 Stomata—The mean and standard deviation of abaxial leaf stomatal density of all plants were $260.45 \pm 112.18 \text{ mm}^{-2}$ (Figure 3.3 A). The mean and standard deviation of abaxial leaf stomatal density of tropical and temperate plants were 257.96 ± 92.14 , and $262.81 \pm 129.55 \text{ mm}^{-2}$ respectively (Figure 3.3 A). The effect of habitat was not significant (Table 3.3). The mean and standard deviation of abaxial leaf stomatal density of low to high elevation were $247.11 \pm 104.91 \text{ mm}^{-2}$, $245.93 \pm 98.75 \text{ mm}^{-2}$, and $280.09 \pm 125.63 \text{ mm}^{-2}$ respectively (Figure 3.3 A). Even though the stomatal density of higher elevation plants tended to be greater than those of low and medium elevation, the effect of elevation on stomatal density was not significant (Table 3.3). However, the interaction between habitat and elevation on stomatal density was significant (Table 3.3). The mean and standard deviation of abaxial leaf stomatal pore length of all plants were $0.019 \pm 0.008 \text{ mm}$ (Figure 3.3 B). The stomatal pore length of tropical plants ($0.022 \pm 0.008 \text{ mm}$) was significantly larger than that of temperate plants ($0.015 \pm 0.006 \text{ mm}$) (Table 3.3). The mean and standard deviation of stomatal pore length of low to high elevation were $0.021 \pm 0.008 \text{ mm}$, $0.021 \pm 0.008 \text{ mm}$, and $0.016 \pm 0.007 \text{ mm}$ respectively (Figure 3.3 B). Even though the stomatal pore length of low and medium elevation plants were greater than that of high elevation, the effect of elevation and the interaction between habitat and elevation on stomatal pore length were not significant (Table 3.3).

The mean and standard deviation of abaxial leaf stomatal pore index of all plants were 0.081 ± 0.048 (Figure 3.3 C). The stomatal pore index of tropical plant (0.100 ± 0.048) was significantly larger than that of temperate plants (0.065 ± 0.043) (Table 3.3). The mean and standard deviation of stomatal pore index of low to high elevation were 0.091 ± 0.036 , 0.091 ± 0.055 , and 0.071 ± 0.044 respectively (Figure 3.3 C). The

stomatal pore index tended to decrease from low to high elevation, but effect of elevation and the interaction between habitat and elevation on stomatal pore index were not significant (Table 3.3).

3.33 Idioblasts—There were no idioblasts found in leaves of the temperate species sampled. Therefore, the test for habitat is not included in the following analysis.

However, based on the sampled accessions in this research, there was a 100% assurance that idioblasts are only found in tropical species. The mean and standard deviation of leaf idioblast density in the tropical accessions were $30.73 \pm 15.26 \text{ mm}^{-2}$ (Figure 3.4 A). The mean and standard deviation of leaf idioblast density from low to high elevation were 36.56 ± 15.97 , 31.66 ± 15.79 , and $21.56 \pm 8.92 \text{ mm}^{-2}$ respectively (Figure 3.4 A). The leaf idioblast density tended to decrease from low to high elevation, but effect of elevation on idioblast density was not significant (Table 3.4). The mean and standard deviation of tropical plant leaf idioblast expression were 9.37 ± 5.53 (Figure 3.4 B). The mean and standard deviation of tropical plant leaf idioblast expression of low to high elevation were 8.00 ± 3.49 , 9.90 ± 5.53 , and 8.84 ± 7.18 respectively (Figure 3.4 B). The effect of elevation on idioblast expression was not significant (Table 3.4).

3.34 Leaf adaxial trichomes—Among all the *Rhododendron* species evaluated in this study, peltate scales (type 2) were most abundant (59.8%). Other types of trichomes were found in comparable frequently between 1.22% - 12.20% of the sampled species. In 4.88% of the samples no epidermal appendages were present (type 1 in Figure 3.5 A).

In temperate plants, peltate scales (type 2) were the most abundant (50.00%). The two least abundant types of trichome were cushion scales (type 3), and dendroid trichomes (type 6). The other trichome types occurred at 5.26% - 10.53% of the sampled

species. In tropical plants, the most abundant trichome also was type 2 (68.18%) and dendroid trichomes (Type 6) were least abundant (6.82%). All the tropical species had trichomes present. Also, hair and multi-branched hair trichomes (types 7, and 8) were absent in tropical plants surveyed (Figure 3.5 B).

In low elevation plants, peltate scales (type 2) were the most abundant (54.55%). Other trichome types (1, 4, 5, 7, and 8) were present on approximately 9% each of the sampled species. Cushion and dendroid trichomes (types 3, and 6) were absent in low elevation plants. In medium elevation plants, peltate trichomes (type 2) were the most abundant (69.44%). Only 2.78% of species sampled from medium elevation lacked trichomes (type 1). Other types of trichome were found in approximately 5.5% of sampled species. Hairs and multi-branched hairs (types 7, and 8) were absent in the medium elevation species. In high elevation plants, peltate scales (type 2) again were the most abundant (50.00%). Cushion scales (Type 3) were least abundant (2.94%). Other trichome types (1, 4, 5, 6, 7, and 8) were found between 5.88 - 8.82% (Figure 3.5 C).

3.35 *The relationship between theoretical specific conductivity and leaf stomatal pore index*—When all species were used in the analysis, there was a significant positive relationship (Table 3.5, Figure 3.6). However, if the analysis was restricted to only temperate species or only tropical species, then the relationship was not significant.

3.4 Discussion

3.41 *General leaf structure*—A wide variation in leaf traits was found for the sampled species even though they were all evergreen shrubs in one subgenus of *Rhododendron*. Evergreen shrubs are generally located at one position (one functional

type) on the LES. The LES suggests that evergreen shrubs have thick, dense leaves that have extended longevity and low gas exchange rates (photosynthesis and transpiration). However, some of the sampled species did have thick succulent leaves with dense mesophyll, yet other sampled species had thinner leaves with a relatively open mesophyll. The adaxial epidermis was most commonly uniseriate; however, in some cases a single hypodermis or multiple hypodermis was present. The most common number of palisade mesophyll layers was two, yet some sampled species had one layer and others three. Many different abaxial appendages were found including scales, hairs and papillae. Moreover, stomatal distribution varied from randomly distributed to completely located within stomatal crypts. Idioblasts were identified in many species. The idioblast central vacuole stained with safranin suggesting that there were phenolic compounds in the lumen of most idioblasts. Some species had idioblast lumens that contained material resembling polysaccharides. No crystals were found in idioblasts suggesting that the idioblasts did not function for calcium management. The sum of the variation in leaf anatomy among leaves of this functional type indicated that leaf anatomical variation ought to be important for adaptation to the climatic and biotic challenges experienced by these species in their native ranges. Moreover, these results indicated that the general LES theory hides important variation in anatomical traits.

3.42 Addressing hypothesis one—Roth (2012) suggested that leaves of tropical species should tend toward hygromorphic leaf traits. If so, then leaves of *Rhododendron* species from tropical habitats should have more hygromorphic leaves than those of *Rhododendron* species from temperate habitats. Hygromorphic leaves tend to be large, thin, have no trichomes, and have fewer but larger stomata (Roth 2012). Leaves of the

selected tropical species in this study were larger than leaves on the selected temperate species, but the difference was not significant. In fact, *Rhododendron* leaves vary in leaf area from 0.1 cm² to 500 cm² across the genus as a whole (Feng 1992). Both the tropical and temperate species sampled had leaf areas in the middle of the range in leaf area for *Rhododendron*, so no significant difference in size would be expected. In fact, in another study, a selection of tropical *Rhododendron* accessions had leaf areas on the smaller side in relation to the genus as a whole (Nilsen et al. 2014). Therefore, there was no support for any difference in leaf area between habitat.

The leaves of the sampled tropical species were significantly thicker than the temperate species. This result is counter to the prediction by (Roth 2012) that tropical leaves have hygromorphic traits. Variation in microhabitat of *Rhododendron* species growing in tropical vs. temperate habitats may have caused the observed difference in leaf thickness. Many tropical *Rhododendron* species at low and middle elevation are epiphytes (Argent 2015), but there are no epiphytic temperate *Rhododendron*. Epiphytes often have relatively thick leaves (for example; orchids) in response to the frequent drought experienced by epiphytes. Therefore, leaf succulence may be an important functional trait for tropical epiphytic *Rhododendron* while not for temperate *Rhododendron* species.

Stomatal density tended to be lower (but not significantly) for sampled tropical plant leaves than that for the sampled temperate leaves as hypothesized by Roth (2012). Also, the stomatal pore length was significantly larger in tropical plant leaves than temperate leaves (Roth 2012). There is a well-documented negative relationship between stomatal density and stomatal pore length (Hetherington and Woodward 2003).

Therefore, the results reflect this negative relationship. Stomatal density of the sampled *Rhododendron* species was in the middle of the domain defined by (Hetherington and Woodward 2003). In fact, the range of stomatal densities for the selected species is a very common range (100 - 400 mm⁻²). However, stomatal pore sizes were relatively small compared to the range presented in (Hetherington and Woodward 2003) and that for *Arabidopsis* for example (Dow et al. 2014).

Leaf potential transpiration is highly related to stomatal pore index (SPI), which is a composite index of stomatal density and pore size (Sack et al. 2003). The general inverse relationship between size and density can result in no difference in stomatal pore index because an increase in density will be associated with a decrease in stomatal pore size. However, the tropical leaves sampled in this study had a significantly higher SPI than the temperate sample, primarily due to larger stomatal pores, suggesting a hygromorphic nature. The comparison of stomatal traits between habitats may have been biased by more high-elevation species in the temperate species selection than the tropical species selected. However, elevation did not have a significant effect on stomatal traits. In fact, stomatal density increased with elevation for temperate species and decreased with elevation for tropical species, which resulted in a significant interaction between habitat and elevation. Moreover, the proportion of species at high elevation in the tropical selection was similar to that in the temperate selection. Therefore, a different distribution of native elevation range between the tropical and temperate samples in this study was not a cause of the habitat effect on SPI.

The presence of cells between the adaxial epidermis and the palisades deserve some comment. A hypodermis is considered a layer of dermal shaped cells below the

epidermis. Several species in this temperate sample contained either a single or multi-layer hypodermis. The hypodermis is thought to function for screening ultraviolet radiation from the palisades cells where much of the leaf photosynthesis occurs. Therefore, the presence of a hypodermis would be anticipated for species residing in high light environments at high elevation. Even though the hypodermis was absent in all tropical plants covered in this research, idioblasts were found, which may have a similar functional significance. Idioblasts were scattered below the epidermis in all the tropical species and were much larger than cells of the hypodermis in temperate species. The fact that the adaxial idioblasts of tropical *Rhododendron* leaves do not usually occur in a complete sheet below the epidermis suggests that they do not necessarily serve as an ultraviolet radiation screening mechanism because lots of gaps in the screen would occur. The large size of the idioblast cells suggests that they may be associated with water storage or water management in the tropical species. If so this would not be in agreement with Roth (2012) because hygromorphic leaves are theorized not to contain any water storage cells. Thus, based on all observations of leaf anatomical traits, tropical *Rhododendron* species did not consistently have more hygromorphic traits than the temperate species sampled in this study thereby rejecting the first hypothesis. Therefore, the tropical leaves tended to be more xeromorphic than temperate leaves.

3.43 Addressing hypothesis two—The second hypothesis suggested that *Rhododendron* leaves trend toward xeromorphy as the native elevation range increases. Xeromorphic leaf traits would include small leaves, succulence, a hypodermis, dense mesophyll, low stomatal pore index, and stomatal crypts (Esau 1960, Roth 2012). Leaf area decreased as the native elevation range increased although leaf form remained

unchanged. Leaf thickness was not affected by elevation, but mesophyll percentage and A_{mes}/A ratio tended to increase with elevation (not significant). In addition, the stomatal pore index was lowest for species with the highest native range elevation. The stomatal pore index tended to decrease from low and medium to high elevation, but the difference was not significant. The likelihood of higher elevation *Rhododendron* leaves to have trichomes was inconclusive, because almost all species included in this study had trichomes regardless of the elevation. Idioblast cells are thought to function in herbivore defense, calcium regulation, or water storage. The idioblast density tended to decrease from low to high elevation tropical *Rhododendron* species, but the difference was not significant. As a result of the data, leaves of *Rhododendron* species show some signs of increasing xeromorphy as the native range elevation increases, but that trend is weak in the sampled species. The influence of elevation on plant traits is usually weak in a multi-species survey because there are many factors that determine the change in climatic traits with elevation and because different mountain ranges may have different patterns of climate change (Körner 2007).

3.44 Addressing hypothesis three—Vascular hydraulic conductivity is coordinated with transpiration in plants (Meinzer et al. 2010). If vascular conductivity is high, more transpiration is allowed. Consequently, a positive correlation between vascular conductivity and transpiration is expected. To address hypothesis three, we looked for a correlation between theoretical specific conductivity data attained in chapter two and stomatal pore index data attained in chapter three. Stomatal pore index (SPI) was positively correlated with stem theoretical specific conductivity (K_s^{theo}) when all data were included in the analysis. However, there was a lot of scatter in the data and the

regression had a low R^2 that was significant only at the 0.1 p -value. Moreover, when the data were separated into temperate and tropical species the correlation was not significant. Perhaps the range of values for theoretical specific conductivity and stomatal pore index were too limited within this single functional group to attain a strong correlation. In fact, the values of both theoretical specific conductivity and stomatal pore index are small relative to other studies on various plants (Hajek et al. 2016, Sack et al. 2005). Because both stomatal pore index and theoretical specific conductivity are small relative to other angiosperm, water flow in *Rhododendron* plants will be low as found previously (Lipp and Nilsen 1997). Plants with low hydraulic conductance also have low productivity (Reich 2014), which reduces potential plant growth rate. Consequently, evergreen *Rhododendron* shrubs in general will not be very competitive in environments where fast growth rate is required for successful colonization of a site (Reich 2014).

3.45 Summary and conclusion—There was wide variation in leaf anatomical traits among species of *Rhododendron*. Also, there was weak support for fundamental trade off suggested by the LES. However, the hypotheses were not well supported because tropical leaves were not strongly differentiated as hygromorphic, and high elevation leaves were not significantly more xeromorphic than low elevation leaves. Variation in plant response to microhabitat (e.g. epiphytic) may have made the expected results for comparison between habitats or along the elevation gradient weak. Overall, leaf anatomical traits of evergreen *Rhododendron* shrubs agree with a slow growth rate and low competitive strength in high resource environments.

3.5 Literature cited

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3.6 Tables

Table 3.1 A list of the *Rhododendron* species used in this research including the native habitat (tropical and temperate) and mean elevation.

Species	Habitat	Average Elevation (m)
<i>R. anthopogon</i>	Temperate	3,127
<i>R. arboreum</i>	Temperate	2,550
<i>R. augustinii</i>	Temperate	2,350
<i>R. calostrotum</i> spp. <i>calostrotum</i>	Temperate	3,962
<i>R. camplyogonum</i>	Temperate	3,508
<i>R. camtschaticum</i>	Temperate	1,600
<i>R. catawbiense</i>	Temperate	1,592
<i>R. cinnabarinum</i>	Temperate	3,127
<i>R. dalhousiae</i> var. <i>rhabdolum</i>	Temperate	2,364
<i>R. dauricum</i>	Temperate	N/A
<i>R. edgeworthii</i>	Temperate	2,898
<i>R. fastigiatum</i>	Temperate	4,038
<i>R. ferrugineum</i>	Temperate	1,900
<i>R. forrestii</i>	Temperate	3,737
<i>R. galactinum</i>	Temperate	3,150
<i>R. hippophaeoides</i>	Temperate	3,335
<i>R. huanum</i> (<i>huianum</i>)	Temperate	2,034
<i>R. hyperythrum</i>	Temperate	1,060
<i>R. Keiskei</i>	Temperate	1,220
<i>R. keysii</i>	Temperate	3,050
<i>R. lepidostylum</i>	Temperate	3,355
<i>R. lindleyi</i>	Temperate	2,745
<i>R. macrophilum</i>	Temperate	600
<i>R. maximum</i>	Temperate	915
<i>R. megeratum</i>	Temperate	3,830

<i>R. minus</i>	Temperate	1,700
<i>R. minus</i> var. <i>chapmanii</i>	Temperate	150
<i>R. orbiculare</i>	Temperate	3,150
<i>R. ponticum</i>	Temperate	900
<i>R. prunum</i>	Temperate	4,130
<i>R. proteoides</i>	Temperate	4,118
<i>R. psuedochrysanthum</i>	Temperate	2,898
<i>R. racemosum</i>	Temperate	3,050
<i>R. ririei</i>	Temperate	1,700
<i>R. rubiginosum</i>	Temperate	3,355
<i>R. sherriffii</i>	Temperate	3,661
<i>R. strigilossum</i>	Temperate	2,828
<i>R. tephropoplum</i>	Temperate	3,370
<i>R. trichostomum</i>	Temperate	3,400
<i>R. williamsianum</i>	Temperate	2,800
<i>R. wolongense</i>	Temperate	1,830
<i>R. aurigeranum</i>	Tropical	1,328
<i>R. blackii</i>	Tropical	2,900
<i>R. bryophilum</i>	Tropical	1,448
<i>R. burtii</i>	Tropical	1,550
<i>R. celebicum</i>	Tropical	1,900
<i>R. correoides</i>	Tropical	3,700
<i>R. crassifolium</i>	Tropical	2,135
<i>R. densifolium</i>	Tropical	1,700
<i>R. goodenoughii</i>	Tropical	1,150
<i>R. gracilentum</i>	Tropical	2,493
<i>R. herzogii</i>	Tropical	2,015
<i>R. himantodes</i>	Tropical	1,650
<i>R. jasminiflorum</i>	Tropical	1,300
<i>R. javanicum</i>	Tropical	1,508
<i>R. kawakamii</i>	Tropical	1,900

<i>R. kochii</i>	Tropical	1,200
<i>R. konori</i>	Tropical	1,625
<i>R. laetum</i>	Tropical	1,968
<i>R. lochiaie</i>	Tropical	1,360
<i>R. longiflorum</i>	Tropical	750
<i>R. loranthiflorum</i>	Tropical	840
<i>R. macgregoriae</i>	Tropical	2,025
<i>R. majus</i>	Tropical	2,885
<i>R. malayanum</i>	Tropical	1,015
<i>R. multicolor</i>	Tropical	1,500
<i>R. orbiculatum</i>	Tropical	1,300
<i>R. polyanthemum</i>	Tropical	1,800
<i>R. praetervisum</i>	Tropical	1,450
<i>R. quadraseanum</i>	Tropical	1,715
<i>R. radians</i>	Tropical	1,600
<i>R. rarilepidotum</i>	Tropical	2,235
<i>R. rarum</i>	Tropical	2,467
<i>R. robinsonii</i>	Tropical	1,432
<i>R. rugosum</i>	Tropical	2,750
<i>R. rushforthii</i>	Tropical	1,650
<i>R. sororium</i>	Tropical	1,550
<i>R. stenophyllum</i>	Tropical	1,950
<i>R. suaveolens</i>	Tropical	1,500
<i>R. superbum</i>	Tropical	2,287
<i>R. taxifolium</i>	Tropical	2,650
<i>R. tuba</i>	Tropical	2,520
<i>R. vaccinioides</i>	Tropical	2,250
<i>R. verticillatum</i>	Tropical	1,100
<i>R. yongii</i>	Tropical	2,490
<i>R. zoelleri</i>	Tropical	675

Table 3.2 Two-way ANOVA table for the effects of habitat, elevation rank, and their interaction on leaf morphology traits of *Rhododendron* species. Habitat = temperate and tropical. Elevation = low (0 - 1,200 m), medium (1,201 - 2,400 m), and high (2,401 - 4,200 m). Area = mean leaf area (cm²); Leaf L/W ratio = mean leaf length per leaf width; Leaf thickness = mean leaf thickness (mm²); % mesophyll = proportion of mesophyll layer thickness per leaf thickness; A_{mes}/A = mean area of the mesophyll per leaf area.

Leaf Morphology-Anatomy	DF	SS	F	Sig.
Leaf Area				
Habitat	1	<0.00	<0.00	0.99
Elevation Rank	2	2128.09	5.41	0.006**
Habitat x Elevation	2	462.57	1.18	0.31
Leaf L/W Ratio				
Habitat	1	1.56	0.24	0.63
Elevation Rank	2	2.11	0.16	0.85
Habitat x Elevation	2	9.13	0.70	0.50
Leaf Thickness				
Habitat	1	0.21	14.47	0.0003***
Elevation Rank	2	0.01	0.45	0.64
Habitat x Elevation	2	0.03	0.88	0.42
% Mesophyll				
Habitat	1	15.09	0.13	0.72
Elevation Rank	2	220.76	0.97	0.38
Habitat x Elevation	2	585.70	2.57	0.0841*

A_{mes}/A				
Habitat	1	23.88	3.47	0.0671*
Elevation Rank	2	5.59	0.41	0.67
Habitat x Elevation	2	35.35	2.57	0.0846*

Table 3.3 Two-way ANOVA table for the effects of habitat, elevation rank, and their interaction on stomatal of *Rhododendron* species. Habitat = temperate or tropical. Elevation = low (0 - 1,200 m), medium (1,201 - 2,400 m), and high (2,401 - 4,200 m). SD = stomatal density (mm^{-2}), SPL = stomatal pore length (μm), SPI = stomatal pore index

Stomata Traits	DF	SS	F	Sig.
SD				
Habitat	1	8895.27	0.75	0.39
Elevation Rank	2	12334.67	0.52	0.60
Habitat x Elevation	2	92263.36	3.87	0.0254**
SPL				
Habitat	1	> 0.00	6.39	0.0138**
Elevation Rank	2	> 0.00	1.44	0.24
Habitat x Elevation	2	> 0.00	0.58	0.56
SPI				
Habitat	1	0.01	5.99	0.0172**
Elevation Rank	2	0.00	0.27	0.76
Habitat x Elevation	2	> 0.00	0.14	0.87

Table 3.4 One-way ANOVA table for the effects of elevation rank on idioblast of *Rhododendron* leaves that have idioblast. Elevation = low (0 - 1,200 m), medium (1,201 - 2,400 m), and high (2,401 - 4,200 m)

Idioblast Traits	DF	SS	F	Sig.
Idioblast Density				
Elevation Rank	2	135.56	0.13	0.88
Idioblast Expression				
Elevation Rank	2	22.65	0.36	0.70

Table 3.5 Regression and correlation results for the relationship between stomatal pore index (SPI) and theoretical specific conductivity (K_s^{theo}) in evergreen species of *Rhododendron*. All = all sampled accessions (n = 84), Temperate = accessions with a native range in the temperate zone (n = 41), Tropical = accessions with a native range in the tropical zone (n = 43)

	Regression R^2	Correlation	Sig.
All	0.061	0.2466	0.0912*
Temperate	0.016	-0.1263	0.4982
Tropical	0.003	-0.0562	0.8303

3.7 Figure legends

Figure 3.1. Representative samples of *Rhododendron* leaves used in this research: (A); images A, B, C, D, E are leaf cross sections captured through a 10 x lens, F - cross section captured through a 4 x lens; (B) Images of abaxial surfaces captured through a dissecting microscope A- peltate scales, B- cuneate scales, C- cushion scales, D- no trichomes, E- hairs, F-Multi-branched hairs; (C) Images of abaxial leaf stomata captured at 40 x, A-stomata among papillae, B- randomly arranged stomata.

Figure 3.2. Box plots of leaf morphological traits for *Rhododendron* accessions used in this research. Each plot has a box plot for the whole data set, for the two habitats (temperate and tropical), and for three elevation ranges (low = 0 - 1,200 m, medium = 1,201 - 2,400 m, high = 2,401 - 4,200 m): (A) Leaf Area (cm^2), (B) Leaf length / leaf width (L/W ratio), (C) Leaf thickness (mm), (D) % of the leaf thickness occupied by spongy mesophyll.

Figure 3.3. Box plots of leaf abaxial stomatal traits for *Rhododendron* accessions used in this research. Each plot has a box plot for the whole data set, for the two habitats (temperate and tropical), and for three elevation ranges (low = 0 - 1,200 m, medium = 1,201 - 2,400 m, high = 2,401 - 4,200 m): (A) Stomatal density (mm^{-2}), (B) Stomatal pore length (μm), (C) Stomatal pore index (SPI = density (length^2)).

Figure 3.4. Box plots of leaf idioblast traits for accessions of *Rhododendron* used in this research that had idioblasts present. Each plot has a box plot for the whole data set and

for three elevation ranges (low = 0 - 1,200 m, medium = 1,201 - 2,400 m, high = 2,401 - 4,200 m): (A) idioblast density ($ID = \# \text{ idioblasts} / \text{length of the section}$), (B) idioblast expression ($IE = ((\sum IA) / \text{total section area})$ where $IA = \text{idioblast area}$).

Figure 3.5. The percentage of the sampled species with each trichome type for; (A) all the species used in this study, (B) separated by habitat type or (C) separated by elevation rank. Trichome types are; 1= no trichomes, 2 = Peltate scale, 3 = Cushion scale, 4 = Cuneate scale, 5 = Stellate scale, 6 = Dendroid scale, 7 = Hair, 8 = multi-branched hair. Elevation ranks = (low = 0 - 1,200 m, medium = 1,201 - 2,400 m, high = 2,401 - 4,200 m).

Figure 3.6. Linear regression between stomatal pore index (dependent variable) and theoretical water transport per area of stems (dependent variable) $SPI = \text{stomatal pore index} = \text{stomatal density} (\text{stomatal pore length}^2)$; Theoretical specific conductivity of stem = $\sum D^4 / A$, where $D = \text{vessel diameter}$ and $A = \text{vascular area}$. Red line represent regression with $R^2 = 0.061$, and $p\text{-value} = 0.0912$

3.8 Figures

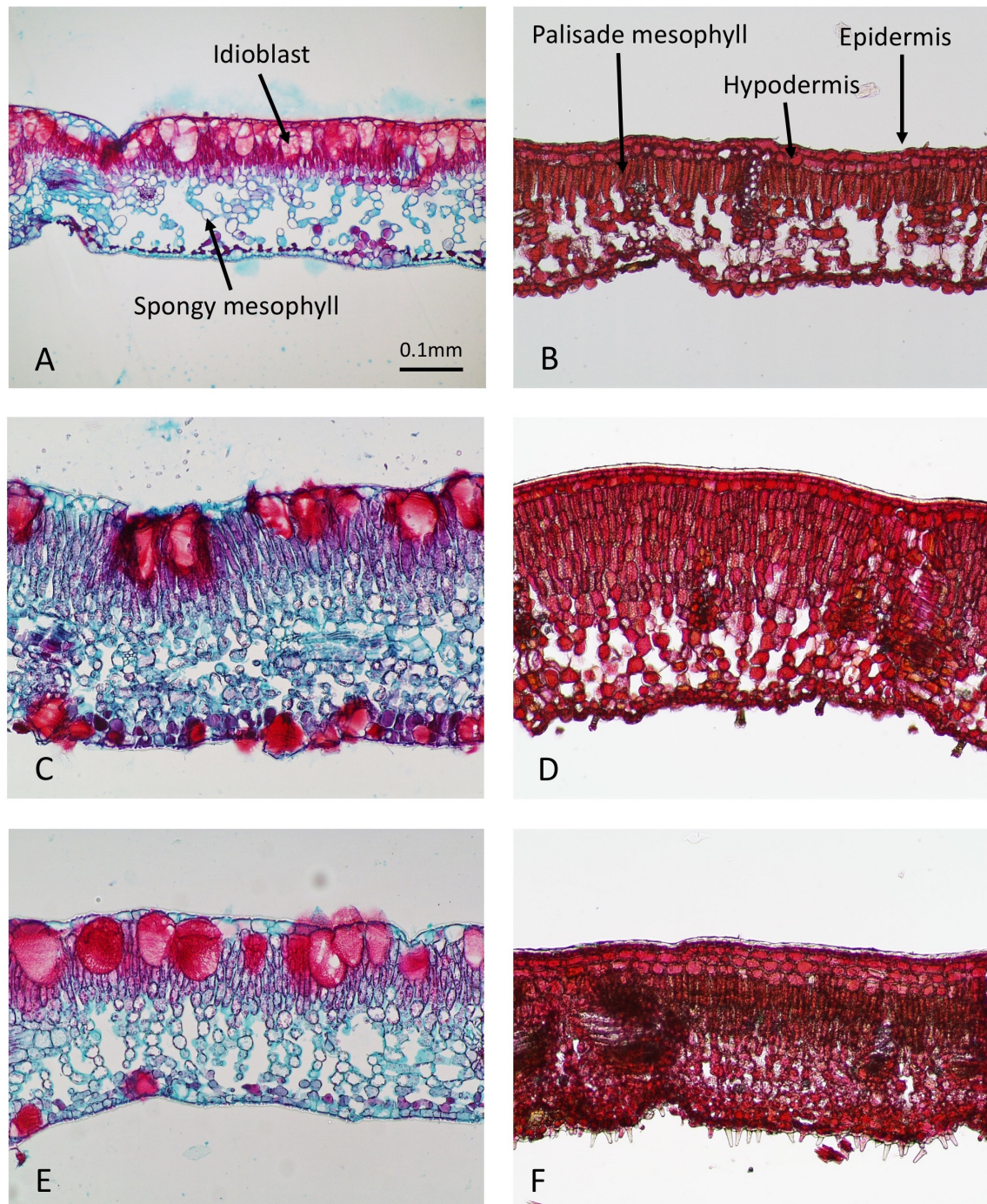


Figure 3.1 A

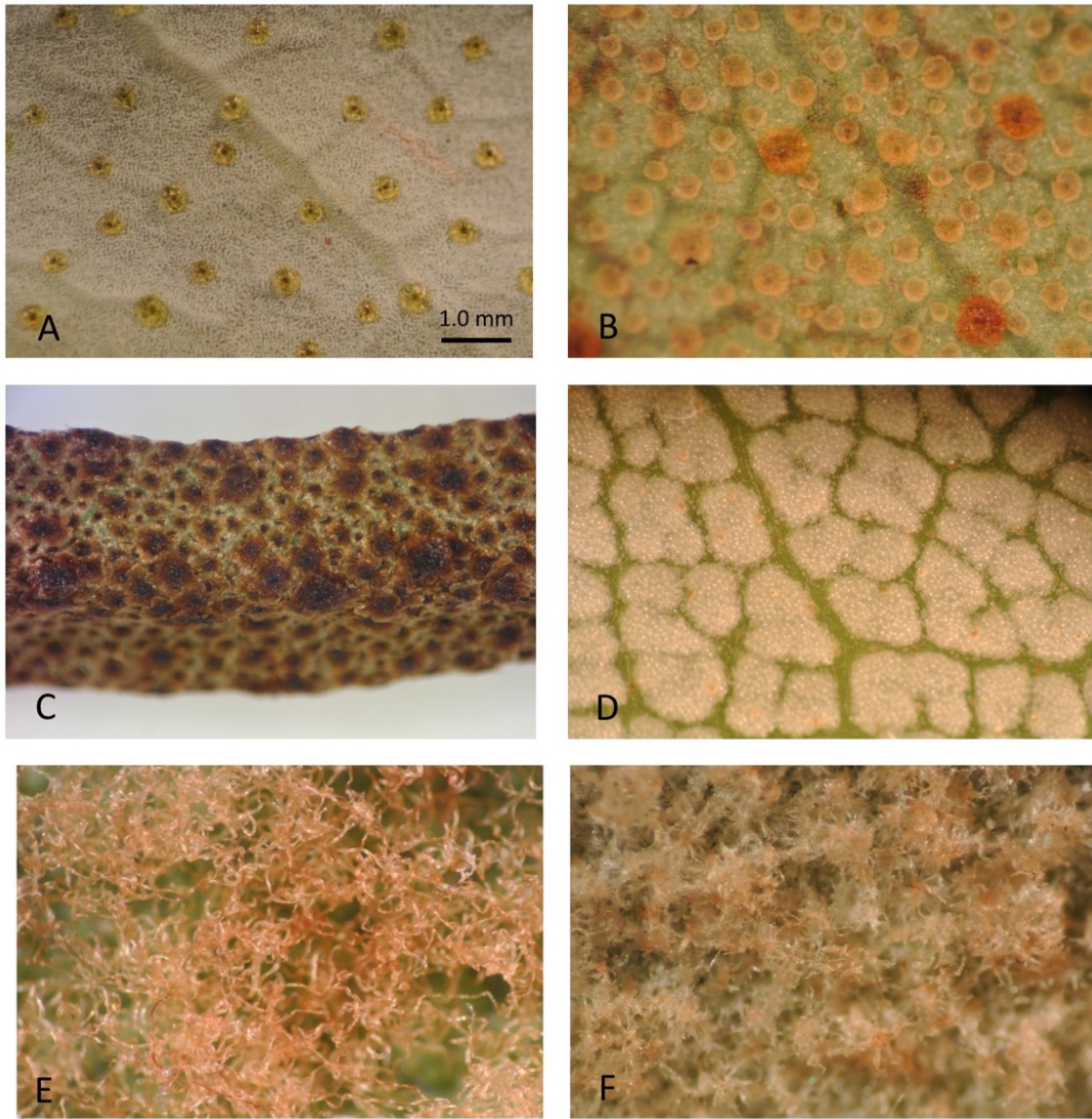


Figure 3.1 B

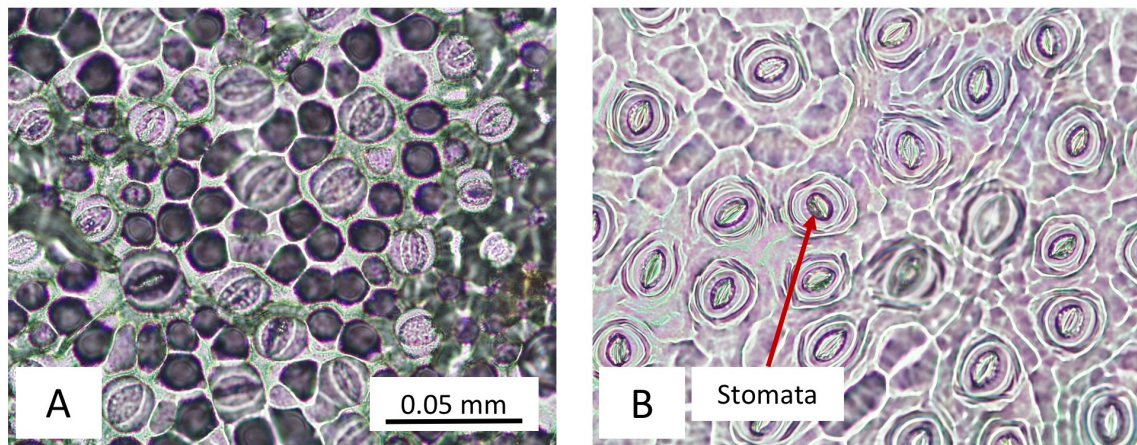


Figure 3.1 C

Figure 3.1. Representative cross sections of *Rhododendron* leaves

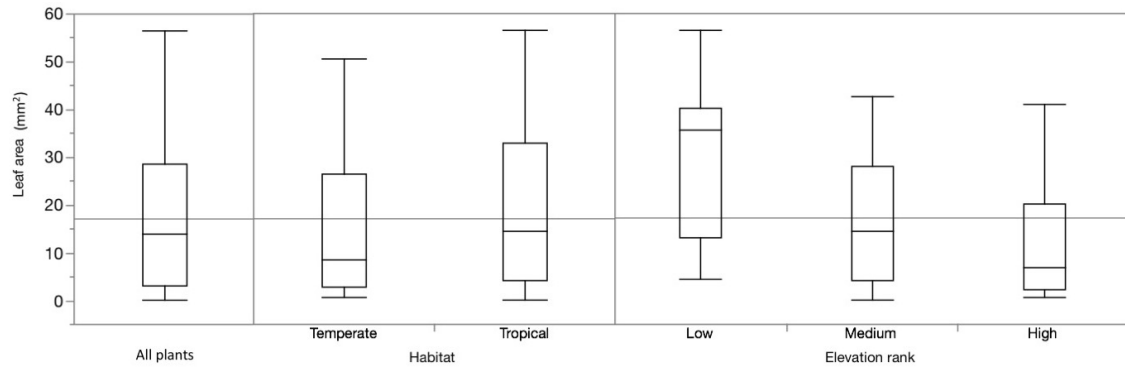


Figure 3.2 A

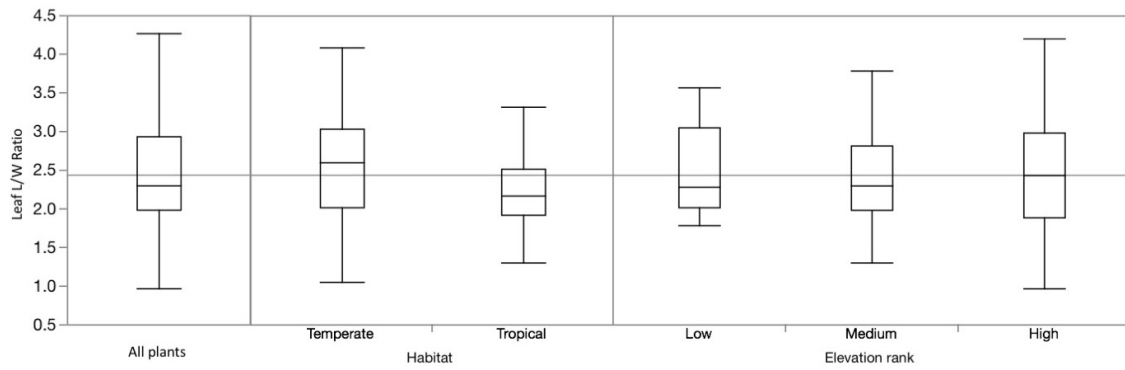


Figure 3.2 B

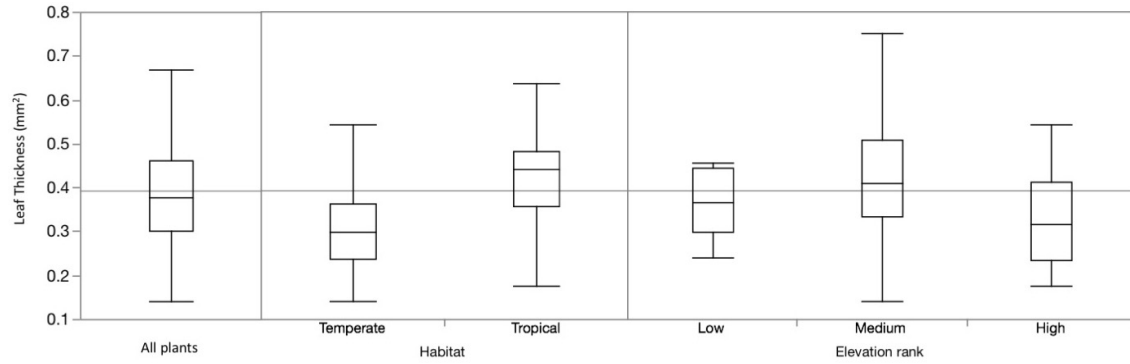


Figure 3.2 C

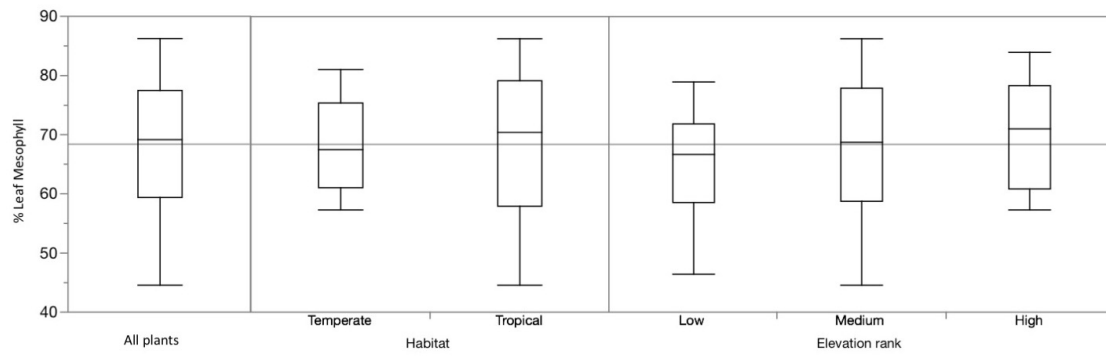


Figure 3.2 D

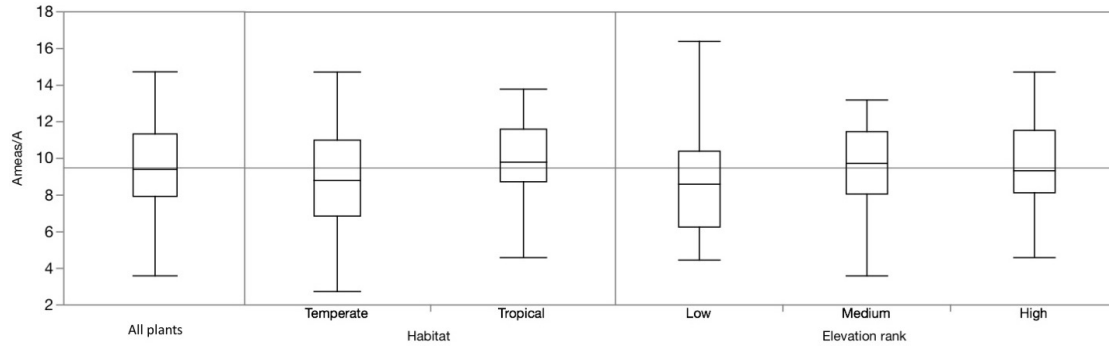


Figure 3.2 E

Figure 3.2. Box plots of leaf morphological traits

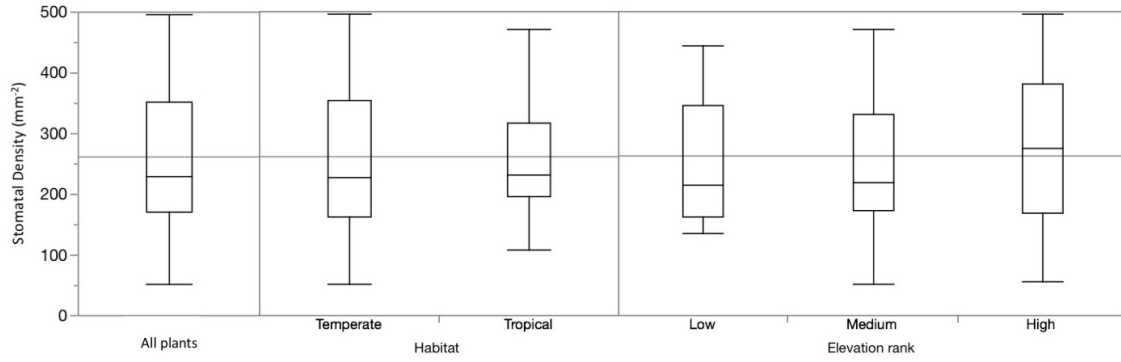


Figure 3.3 A

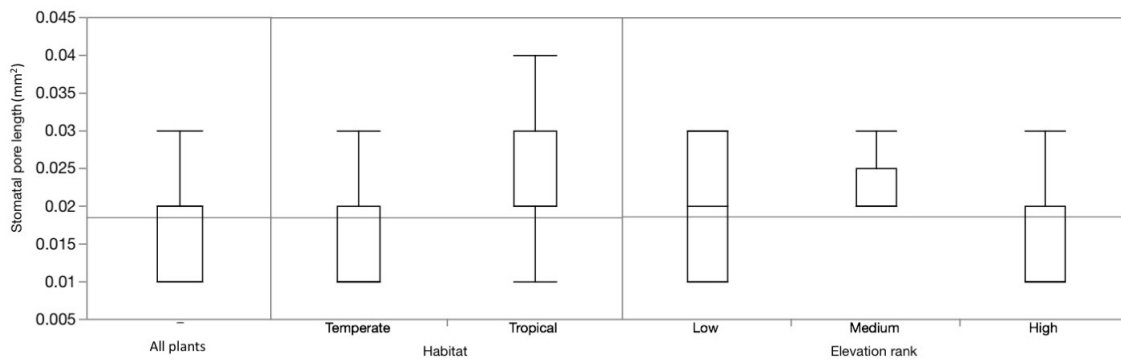


Figure 3.3 B

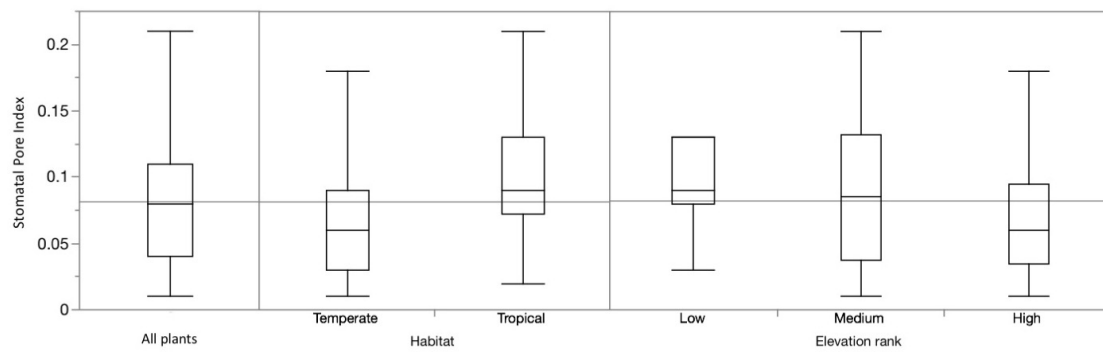


Figure 3.3 C

Figure 3.3. Box plots of leaf abaxial stomatal traits

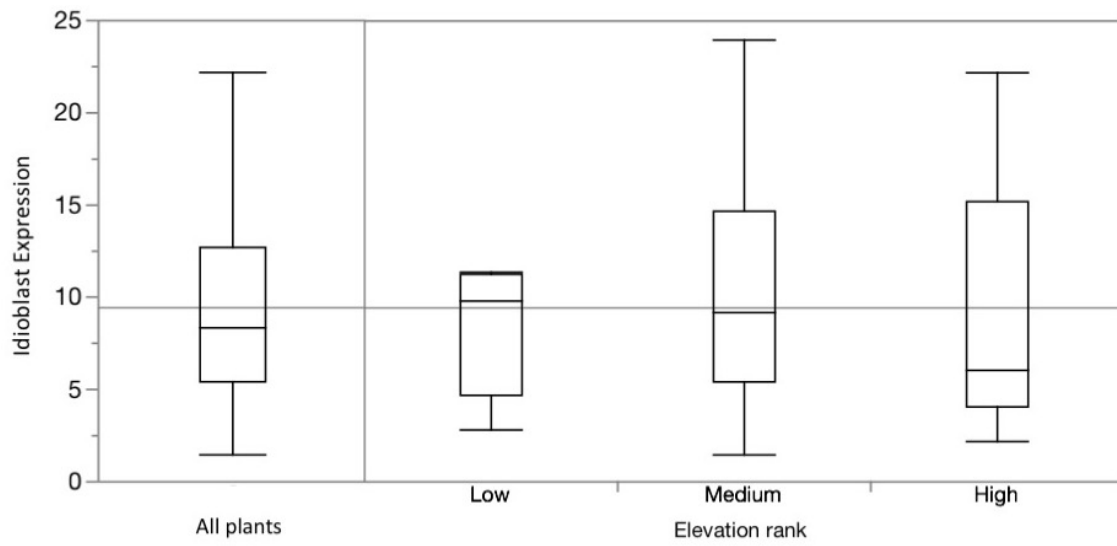


Figure 3.4 A

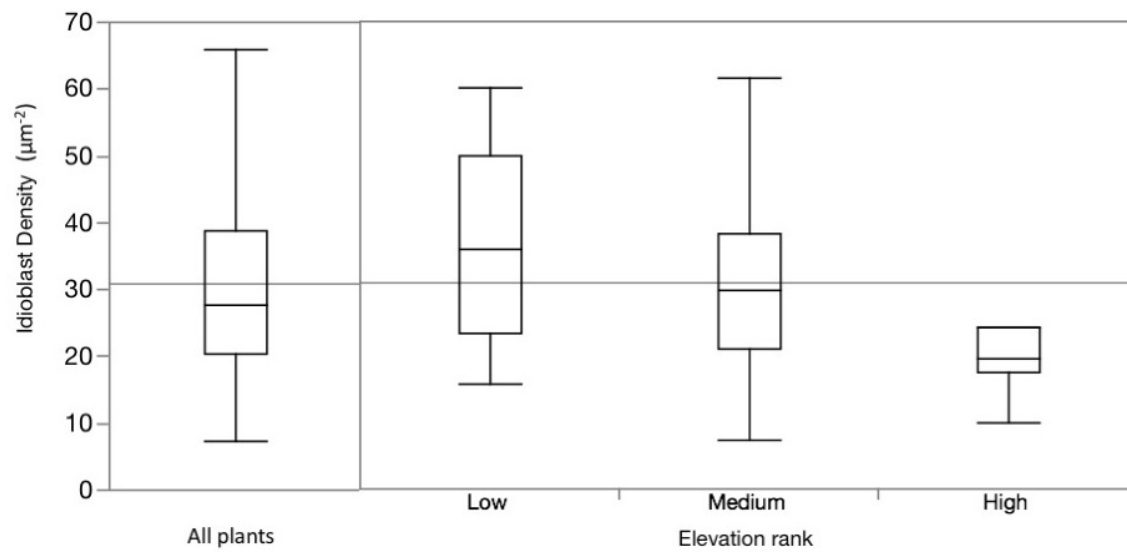


Figure 3.4 B

Figure 3.4. Box plots of leaf idioblast traits

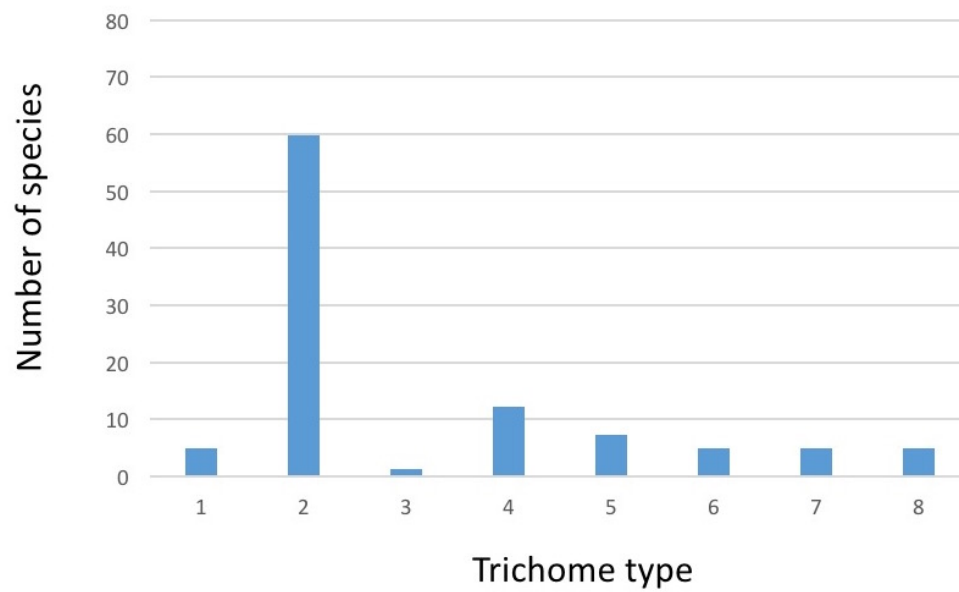


Figure 3.5 A

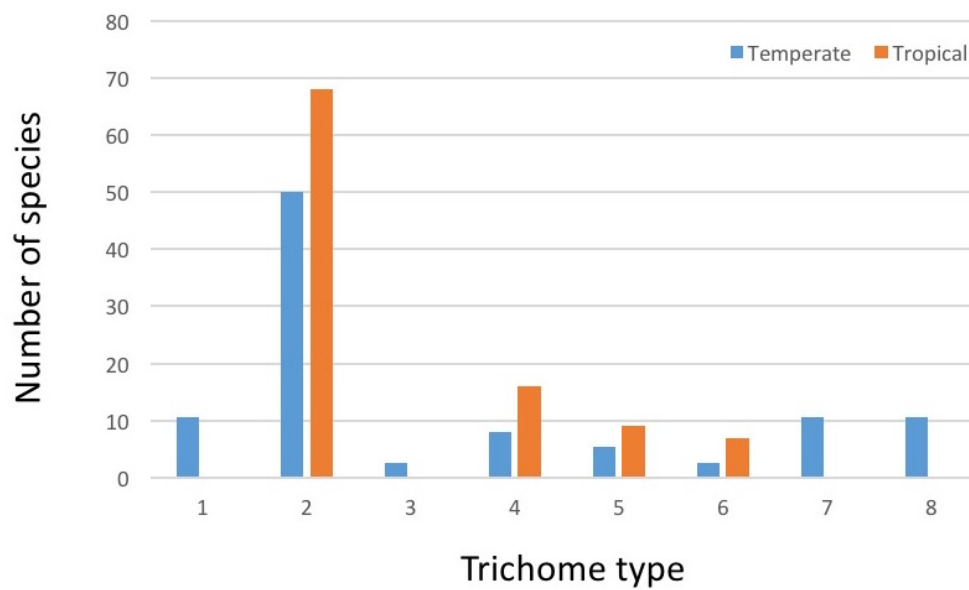


Figure 3.5 B

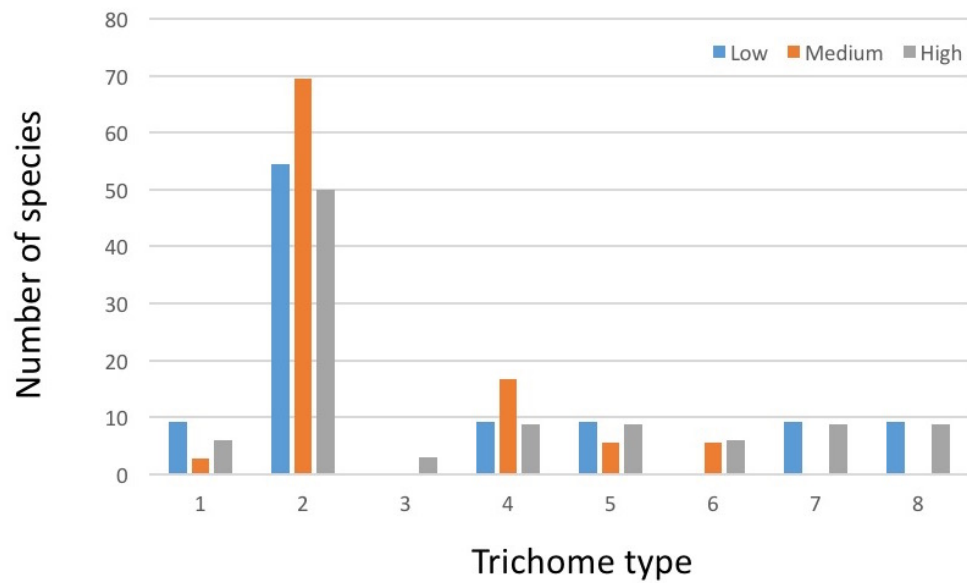


Figure 3.5 C

Figure 3.5 Distributions of trichome types

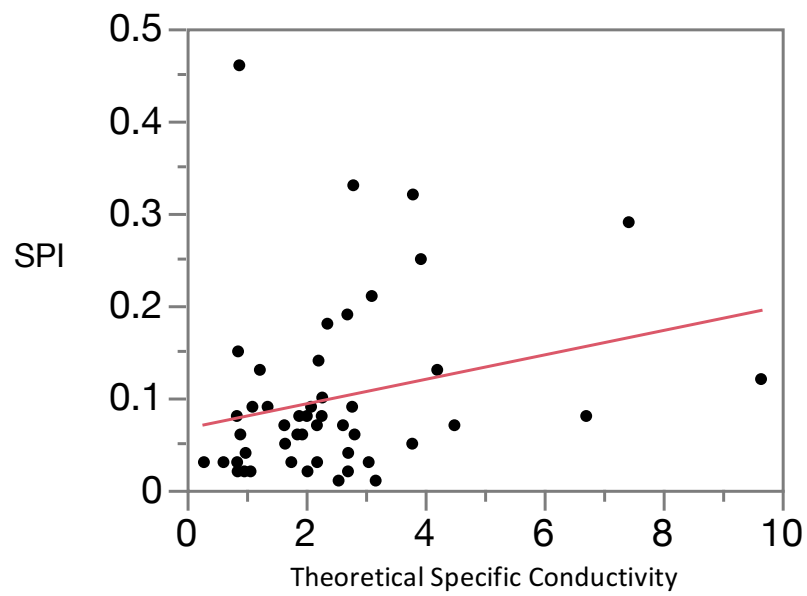


Figure 3.6. Regression plot for the relationship between SPI and K_s^{theo}

CHAPTER 4

The significance of idioblasts to bulk leaf water relations in tropical accessions of

Rhododendron

4.1 Introduction

Idioblasts are individual cells that are distinctly different in form (usually much larger) than the surrounding cells of a tissue (Esau 1960). In general, idioblasts can be found in all plant tissues and are structurally diverse. Some idioblasts are sclereids, others are secretion cells and others are large storage cells with little or no cytoplasm (Esau 1960). Many idioblasts contain substances such as oils, tannins, mucilage, or calcium oxalate crystals. These differences in structure and content of idioblasts among species have been used for taxonomic purposes (Lersten and Curtis 1995). Foliar idioblasts are found in many plant families including the Alsteromeriaceae (Lyshede 2002), Brassicaceae (Andreasson et al. 2001), Ericaceae (Nilsen and Scheckler 2003), Lamiaceae (Lersten and Curtis 1998), Papilionaceae (Sartori and Tozzi 2002), Polygonaceae (Lersten and Curtis 1992), Scrophulariaceae (Lersten and Curtis 2001) Sterculiaceae (Lersten and Curtis 1997b) and Solanaceae (Leite et al. 1999) to name a few. Thus, idioblasts are diverse, widely distributed among plant taxa and significant for taxonomic purposes. However, the relationship between idioblast cells and leaf physiological properties remains largely unexplored (Körner et al. 1983, Nilsen and Scheckler 2003).

The predominant hypotheses for the functional significance of idioblasts are; secretion (Feio dos Santos et al. 2013), storage of cellular waste products or crystals

(Franceschi and Nakata 2005), or defense against herbivores (Leite et al. 1999, Andreasson et al. 2001, Thangstad et al. 2004). Yet, a large quantity of idioblast cells in a leaf could have several simultaneous different effects on leaf physiology. For example, a large proportion of leaf water contained in idioblasts could affect bulk leaf osmotic potential, capacitance and hydraulic conductance. Also, sub-epidermal idioblasts that extend into the palisade cell layer could significantly alter the dynamics of light penetration into leaves and thereby influence leaf absorptivity of light and photosynthetic response to light (Karabourniotis 1998, Nilsen and Scheckler 2003). Moreover, if idioblasts increase the ratio of water to air in a leaf then the thermal dynamics of the leaf will change perhaps buffering the rate by which leaf temperature changes during and after a sun exposure. In order to understand the broad significances of idioblasts to leaf physiology, the relationships between idioblast abundance, lamina anatomy, morphology, and water relations should be understood.

Foliar idioblasts in *Rhododendron* subgenus *Vireya* are excellent models for studying the relationship between idioblast abundance and leaf water relations. All previously surveyed species of *Rhododendron* in subgenus *Vireya* possess idioblasts (Chapter 3; *R. saxifragoides* is an exception), but idioblasts have not been found in other subgenera of *Rhododendron* (Körner et al. 1983, Nilsen and Scheckler 2003, Argent 2006). Species in subgenus *Vireya* are found in Malesia, which is a tropical environment. Malesia is the region between Malaysia and Papua New Guinea. Idioblasts on the adaxial side of leaves are the most abundant, but abaxial idioblasts can be found clustered around the base of scales (Nilsen and Scheckler 2003). The abundance, size and expression (total leaf volume occupied by idioblasts) may vary greatly among species of subgenus *Vireya*

(Nilsen 2003). However, no crystalline structures have been found in idioblasts of *Vireya* species, which rules out the potential function as calcium oxalate crystal storage. Adaxial idioblasts in subgenus *Vireya* leaves contain primarily water, but can contain oil droplets, mucilage, and compounds containing phenol groups (positive Safranin O response). The large water content of these cells suggests that idioblasts may have an impact on bulk leaf lamina water relations of species in subgenus *Vireya*, which are tropical species.

The overall goal of this study was to survey idioblast expression (proportion of leaf lamina occupied by idioblasts) among accessions of *Rhododendron* species in subgenus *Vireya* and utilize a gradient in idioblast expression among accessions to evaluate the relationships between idioblast expression, leaf morphology, leaf anatomy, and bulk leaf water relations traits. The following specific hypotheses were addressed in this study.

1) There are significant differences in idioblast expression among accessions of subgenus *Vireya*. Significant differences in idioblast expression among accessions need to be demonstrated before hypotheses about functional significance can be posed.

2) Idioblast expression increases in accordance with an increase in leaf succulence among plants. Idioblast expression is a main factor defining leaf succulence because of the large cells and their water content. We suggest that the greater the proportion of large water filled cells in the leaf lamina the larger the amount of water in the lamina (leaf succulence) on an area basis.

3) Stomatal pore index (SPI), a proxy for maximum stomatal conductance, increases in accordance with an increase in idioblast expression among plants. This third hypothesis is based on the idea that the larger the volume of total idioblasts in leaf the

larger will be the water resource for the leaf. Also, if there is a large water resource in the leaf, leaf conductance can be higher allowing for a higher stomatal pore index.

4) Leaf capacitance, before the turgor loss point (pre-TLP), increases in accordance with an increase in leaf idioblast expression among plants. This fourth hypothesis is based on the concept that a large volume of total idioblast cells will allow leaf to lose a large amount of water with only a moderate change in bulk leaf water potential.

This study provides important information about the relationships between leaf idioblast expression, leaf anatomical traits, leaf morphological traits, and leaf water relations components. This research documents significant differences in idioblast expression among species and shows that idioblasts increase leaf succulence and leaf capacitance for relatively thin leaves.

4.2 Materials and methods

4.21 Plant growth conditions—In order to determine the relationship between idioblast expression and leaf water relations, 61 plants representing 17 species from subgenus *Vireya* were grown on a greenhouse bench in the Biological Sciences-VBI Plant Growth Facility (VT-PGF), Virginia Tech. The accessions were obtained from the Rhododendron Species Foundation and Botanical Garden, Federal Way, WA. The growth medium used in the greenhouse experiment was a mixture of coconut chunks (70%) and perlite (30%). Plants were watered daily and fertilized every two to three weeks with a balanced liquid fertilizer (Miracle-gro; water soluble azalea, camellia, *Rhododendron* plant food; 30% N: 10% P: 10% K). Only leaves produced in the Biological Sciences-

VBI Plant Growth Facility (VT-PGF) were used in the greenhouse experiment. Air temperature in the greenhouse ranged from 22 – 32 °C across the year and relative humidity ranged from 65% to 100% on a daily basis.

4.22 Leaf collection — Six to eight fully mature leaves were collected from the four major compass directions from outer canopy locations of each plant. The leaves were sealed in zip-lock plastic bags and transported to the lab for morphological and anatomical analyses. Each set of leaves was placed abaxial side down on a scanner next to a ruled paper (cm) for scale. The scans at 600 dpi were stored in JPEG format for later analysis of leaf morphology. Leaf length, width and area were determined with ImageJ (National Institutes of Health (NIH), Bethesda, MD). Leaf length divided by leaf width was used as an index of leaf lamina shape (high value = linear, low value = ovoid).

4.23 Leaf anatomy—The abaxial surface of three randomly selected recently mature leaves from the outer canopy was painted (between two main lateral veins) with clear nail polish (model 504; Clairol Inc., Stamford, CT). When the polish was dry and hardened, a narrow piece of clear adhesive tape (Scotch brand package sealing tape) was placed over the nail polish, pressed down firmly, carefully lifted to remove the dried sheet of nail polish (the peel), placed (with the peel facing upward) on a microscope slide and covered with a cover-slip. Five randomly located images (25 x objective lens) of the epidermal peels were captured with a digital camera (Olympus DP-10; Olympus Optical Co. Ltd., Tokyo, Japan) mounted on a bright field microscope (Olympus BX50; Olympus Optical Co. Ltd., Tokyo Japan). All stomata were counted in the field of view of each image to determine stomatal density. Five additional images (40 x objective lens) were captured from the same epidermal peel for stomatal size. The length of every stomatal

pore and the total number of pores in the field of view were determined using ImageJ software. The stomata pore index (SPI) was calculated for each image as: $D(L^2)$. Where D = stomata density, and L = mean stomatal pore length (Parlange and Waggoner 1970).

Following the removal of nail polish, five sections (each 3 mm in width) from the mid-vein to the margin were excised from each leaf (avoiding the nail polish affected area) and preserved in FAA (50% ethanol, 35% water, 10% formalin, 5% glacial acetic acid). Following complete preservation (approximately 1 week), sections were sequentially hydrated to remove the FAA, dehydrated in an alcohol gradient (5 steps at 30 minutes each), saturated with xylene (two 1 hr soaks), impregnated with paraffin oil (1 step with 50% paraffin oil and xylene for 1 hr plus 2 steps of 1 hr each of pure paraffin oil), and equilibrated in hot paraffin (3 steps of 8 hr each). The leaf pieces were trimmed and mounted in paraffin for laminal cross sections (8 μ m) made with a rotary microtome (model HN 340E; Microm International GmbH, Walldorf, Germany). One ribbon (8-10 cross sections) was mounted from one of the leaf pieces from each leaf. The resulting tissue sections were double stained with safranin O and fast green before permanent mounting with Permounttm. Digital images were taken (10 x objective lens) from the best section on each slide (Figure 4.1). The length and width was measured for each idioblast in each image. The density of idioblasts per leaf length (mm) was calculated. Also, the area of each idioblast was calculated by using the geometric formula for the area of an ellipse ($IA = \pi ab$), where a = length and b = width (Selby 1972). The IAs were summed and divided by the total area of the section to attain an index of idioblast expression ($IE = ((\Sigma IA) / \text{total section area}) \times 100$). In addition, average leaf thickness and % of leaf area occupied by spongy mesophyll were recorded for each image.

4.24 Moisture release curves—Three recently mature leaves were excised from each plant in the greenhouse experiment in early morning (before 7:00AM) and placed in a saturation chamber to become fully saturated with water. Following saturation the relationship between water deficit and equilibrium pressure (model 1000, PMS Inc, Corvallis, OR) was determined using a dehydration technique (Sperry and Sullivan 1992, Sperry and Saliendra 1994). A graphical technique was used to determine osmotic potential (OP) relationships, water deficit at TLP, and leaf capacitance pre- and post-TLP. Bulk leaf capacitance (C_{bulk}) was expressed in absolute terms and normalized by leaf anatomy ($\text{Mol m}^{-2} \text{MPa}^{-1}$) as done previously (Brodribb and Holbrook 2003, Blackman and Brodribb 2011). Succulence of every leaf was determined as the total grams of water per leaf area (g cm^{-2}) in the fully saturated leaf ((weight at full saturation – dry weight) / leaf area).

4.25 Data analysis and statistics—Plant ($n = 61$) was the experimental unit for leaf anatomical and water relations traits in the greenhouse study. The effect of species ($n = 3$ to 6 plants species⁻¹) on anatomical or water relations traits were determined by one-way analysis of variance (ANOVA). Spearman correlations were made between all trait variables for plants. The relationships between anatomical traits and water relations traits for plants were determined by regression analysis. The best model to describe leaf idioblast expression in plants based on leaf anatomical traits was selected by stepwise analysis (forward, backward or mixed). A similar procedure was used to find the best model that described the pre- and post-turgor loss capacitance. All statistical analyses were performed using JMP Pro 12 (SAS Institute Inc., Cary, NC).

4.3 Results

4.3.1 Leaf anatomy—There was a highly significant effect of species on all measured anatomical and morphological traits (Table 4.1). All stomatal traits were significantly different among species. Leaf area ranged from $56.4 \text{ cm}^2 \pm 4.14$ for *R. zoelleri* to $8.4 \text{ cm}^2 \pm 0.73$ for *R. bryophyllum*. Leaf shape (leaf L/W ratio) was most linear for *R. bryophyllum* (3.1 ± 0.18) and most round for *R. orbiculatum* (1.3 ± 0.21). The thickest leaves were those of *R. yongii* ($0.828 \text{ mm} \pm 0.071$) and the thinnest leaves were those of *R. celebicum* ($0.344 \text{ mm} \pm 0.053$). The least succulent leaves were those of *R. multicolor* ($0.023 \text{ g cm}^{-2} \pm 0.002$) while those of *R. yongii* had the highest succulence ($0.057 \text{ g cm}^{-2} \pm 0.001$). *R. crassifolium* had the highest percent of leaf thickness taken up by spongy mesophyll ($68.3 \% \pm 2.77$) while that of *R. multicolor* was the lowest ($44.6 \% \pm 3.78$). Idioblast density was the highest for *R. celebicum* ($66.0 \text{ mm}^{-2} \pm 20.87$) and lowest for *R. yongii* ($23.4 \text{ mm}^{-2} \pm 2.53$). *R. bryophyllum* had the largest average idioblast size ($3.33 \text{ } \mu\text{m}^2 \pm 0.306$) while that of *R. kochii* were the smallest ($1.01 \text{ } \mu\text{m}^2 \pm 0.039$). Idioblast expression was highest for *R. bryophyllum* ($19.3 \% \pm 1.20$) and lowest for *R. kochii* ($4.7\% \pm 0.96$). Stomatal density ranged from $470 \text{ mm}^{-2} \pm 43.7$ for *R. rarilepidotum* to $171 \text{ mm}^{-2} \pm 45.8$ for *R. celebicum*. Stomatal pore length was largest for *R. orbiculatum* ($0.039 \text{ mm} \pm 0.001$) and smallest for *R. kochii* ($0.014 \text{ mm} \pm 0.001$). Stomatal pore index (SPI) was the highest in *R. orbiculatum* (0.334 ± 0.171) and the lowest in *R. javanicum* and *R. crassifolium* (0.076 ± 0.009).

4.3.2 Water relations—There was a significant species effect on all water relation traits except the osmotic potential at full turgor minus the osmotic potential at zero turgor (Table 4.2). The effect of species on the OP at the TLP had the highest significance

among all the water relations traits. Bulk leaf capacitance pre TLP was always less than bulk leaf capacitance post TLP. Bulk leaf capacitance pre TLP was highest for *R. crassifolium* ($2.17 \text{ Mol m}^{-2} \text{ MPa}^{-1} \pm 0.68$) and lowest for *R. kochii* ($0.68 \text{ Mol m}^{-2} \text{ MPa}^{-1} \pm 0.08$). However, bulk leaf capacitance post TLP was highest for *R. orbiculatum* ($5.92 \text{ Mol m}^{-2} \text{ MPa}^{-1} \pm 0.0129$) and lowest for *R. kochii* ($1.63 \text{ Mol m}^{-2} \text{ MPa}^{-1} \pm 0.107$). The water deficit at the TLP ranged from $14.2 \% \pm 1.56$ for *R. bryophyllum* to $6.44 \% \pm 1.19$ for *R. goodenoughii*. The OP at full turgor was lowest for *R. robinsonii* ($-1.41 \text{ MPa} \pm 0.070$) and highest for *R. bryophyllum* ($-0.83 \text{ MPa} \pm 0.12$). Also, *R. robinsonii* had the lowest OP at the TLP ($-1.78 \text{ MPa} \pm 0.129$), and the highest OP at the TLP was for *R. aurigeranum* ($-1.20 \text{ MPa} \pm 0.549$). The largest difference between the OP at full turgor and the OP at the TLP was for *R. aurigeranum* ($0.51 \text{ MPa} \pm 0.066$), while the smallest value for this trait was $0.29 \text{ MPa} \pm 0.139$ for *R. crassifolium* leaves.

4.33 Correlations—Spearman correlations were performed between all traits resulting in an 18 by 18 matrix of correlation coefficients with many cases of autocorrelation. In order to highlight the important relationships from this matrix and to avoid showing autocorrelations, only the significant correlations for the focus traits of this study are presented in Table 4.3. Idioblast density was significantly, positively correlated with leaf L/W ratio which indicated that idioblast density increased as leaves became thinner and longer. In addition, idioblast density was significantly negatively correlated with leaf thickness, leaf succulence and % mesophyll. All three of these anatomical traits were highly autocorrelated and represent an axis of leaf succulence. Therefore, idioblast density decreased as leaf lamina became more succulent. Idioblast expression was similarly correlated with anatomical traits as that of idioblast density.

Stomatal density on the abaxial lamina surface was significantly, negatively correlated with both idioblast size and idioblast expression (Table 4.3). Therefore, stomatal density decreased as idioblast expression increased. Also, stomatal density was significantly, negatively correlated with leaf area and leaf length (leaf length was autocorrelated with leaf area). Thus, stomatal density decreased as leaf area increased. Unlike stomatal density, stomatal pore length and SPI were significantly, positively correlated with leaf thickness and leaf succulence. Therefore, this result suggested that SPI, a proxy for maximum leaf conductance, increased as leaf succulence increased and that increase was primarily due to increasing stomatal pore length.

Bulk leaf capacitance pre-TLP was significantly, negatively correlated with leaf area traits (area and length) and significantly, positively correlated with leaf L/W ratio (Table 4.3). Thus, smaller leaves that are more linear in shape had higher bulk leaf capacitance pre-TLP than larger more lanceolate leaves. Also, leaf capacitance pre-TLP was significantly, positively correlated with all three traits of the leaf succulence axis indicating that leaves with greater succulence had a larger bulk leaf capacitance pre-TLP. In contrast, capacitance post-TLP was not significantly correlated with leaf area but was significantly, negatively correlated with leaf L/W ratio. Therefore, bulk leaf capacitance post-TLP was larger in lanceolate leaves in comparison with more linear leaf shapes. In accordance with the results for bulk leaf capacitance pre-TLP, the bulk leaf capacitance post-TLP was significantly, positively correlated with the three leaf succulence traits. Thus, both bulk leaf capacitance pre- and post-TLP were larger in more succulent leaves. Also, bulk leaf capacitance post-TLP was significantly, positively correlated with idioblast expression and idioblast density (Table 4.3). This result indicates that leaves

with higher idioblast expression had a larger bulk leaf capacitance post-TLP. Moreover, bulk leaf capacitance post-TLP was significantly, positively correlated with SPI. The significance of this correlation was relatively weak compared with the other significant correlations of anatomical traits with bulk leaf capacitance post-TLP.

The WD at TLP was significantly, negatively correlated with leaf area traits, but significantly, positively correlated with leaf L/W ratio (Table 4.3). Therefore, the WD at TLP was higher for smaller more linear leaves than for larger more lanceolate leaves. The WD at TLP was significantly, positively correlated to idioblast expression and idioblast density. Thus, leaves with higher idioblast expression also had a higher WD at the TLP. In addition, the WD at TLP was significantly, positively correlated with both pre- and post-TLP bulk leaf capacitance. Therefore, the WD at TLP increased in coordination with an increase in bulk leaf capacitance.

The OP at 100% (full turgor) was significantly, positively correlated with leaf area and length and was significantly, negatively correlated with leaf L/W ratio (Table 4.3). Therefore, OP at 100% became progressively more negative as leaves became larger and more lanceolate. In addition, the OP at 100% was significantly, negatively correlated with pre-TLP bulk leaf capacitance and significantly, positively correlated with post-TLP bulk leaf capacitance.

The OP at TLP was significantly, positively correlated with leaf area and significantly, negatively correlated with leaf L/W ratio. Thus, similarly to OP at full turgor, the OP at TLP was higher in larger and more lanceolate leaves. There were no significant correlations of either OP or leaf succulence traits. However, OP at TLP was

significantly, negatively correlated with capacitance post-TLP. Therefore, leaves with a highly negative OP at TLP will tend to have low post-TLP capacitance.

4.34 Regressions—The regression of capacitance pre-TLP as dependent variable against idioblast expression as independent variable was not significant ($R^2 = 0.01845$, $p = 0.7836$). If the data for plants with leaves thicker than 0.5 mm are culled from the data set ($n = 32$ remaining), then there is a significant regression with a positive slope at the $\alpha = 0.1$ level (adjusted $R^2 = 0.1002$, $p = 0.0723$) between capacitance pre-TLP as dependent variable and idioblast expression as independent variable (Figure 4.2 A). However, when all plants are involved in the regression, there was a significant regression (adjusted $R^2 = 0.1531$, $p = 0.0024$), with a negative slope, for capacitance post-TLP as dependent variable against idioblast expression as independent variable (Figure 4.2 B). If the data for plants with leaves thicker than 0.5 mm are culled from the data set, then there is still a significant regression (adjusted $R^2 = 0.1712$, $p = 0.0167$), with a negative slope, between capacitance post-TLP as dependent variable and idioblast expression as independent variable.

There was a significant positive regression (adjusted $R^2 = 0.1106$, $p = 0.0115$) between WD at TLP as independent variable against idioblast expression as dependent variable (Figure 4.2 C).

4.35 Stepwise and regression models—The best model for leaf idioblast expression using leaf lamina anatomical traits that was developed by stepwise analysis (forward, lowest BIC) was defined by the leaf shape and leaf succulence traits: leaf L/W ratio ($p = < 0.0001$), % mesophyll ($p = < 0.0001$). The resulting two variable model defined over 54% of the variation in idioblast expression among plants (adjusted $R^2 =$

0.5960, minimum BIC = 291.3). The best model for leaf idioblast expression based on water relations traits (including stomatal traits) was a three variable model including WD at TLP ($p = 0.0010$), capacitance post-TLP ($p = < 0.0001$) and OP 100% - OP at TLP ($p = 0.0141$). The resulting model defined 48% of the variance in idioblast expression among plants (adjusted $R^2 = 0.4930$, minimum BIC = 274.8).

The best model (stepwise, forward) for stomatal pore index based on anatomical traits was defined by only one variable, leaf thickness ($p = 0.0035$), which accounted for 55% of the variance in SPI (adjusted $R^2 = 0.1448$, minimum BIC = -69.99). The best model (stepwise, forward) for SPI based on water relations traits alone was also defined by one variable, capacitance post-TLP ($p = 0.0407$). The resulting model defined 50% of the variance in SPI among plants (adjusted $R^2 = 0.0627$, minimum BIC = -59.19).

The best model (stepwise, forward) for capacitance pre-TLP using all anatomical traits including stomatal traits was a three variable model including leaf succulence ($p = 0.0002$) leaf area ($p = 0.0003$) and leaf L/W ratio ($p = 0.0450$). The resulting model defines 48% of the variance in pre-TLP capacitance (adjusted $R^2 = 0.4394$, minimum BIC = 63.67). The best model (stepwise, forward) for capacitance post-TLP using all anatomical traits including stomatal traits was a one variable model using % mesophyll ($p = 0.0001$), which defined 50% of the variation of post-TLP capacitance (adjusted $R^2 = 0.2555$, minimum BIC = 203.39).

4.4 Discussion

4.41 General anatomical characteristics of the *Rhododendron* leaf—Leaf area of tropical *Rhododendron* species in subgenus *Vireya* used in this study tended to be at the

smaller range of leaf sizes of *Rhododendron* species in a broad sense (Nilsen et al. 2014). The mean leaf area and shape (leaf L/W ratio) of the species used in this study fell into the range of that for other species in subgenus *Vireya*. There are a few (11 out of 323 species in total) species in subgenus *Vireya* (Argent 2015) with particularly narrow leaves ($L/W > 5$). No species used in this study fell into the category of narrow leaves for subgenus *Vireya*. Therefore, the accessions used represent species with lanceolate and ovoid leaf form. Leaf thickness of this sampled taxa included the entire range of leaf thickness variation (0.25 mm to 0.95 mm) for leaves in subgenus *Vireya* (personal observation). The leaf lamina of *Rhododendron* species tends to be rigid even after turgor loss while the petiole tends to be flexible, particularly during water limitation. Therefore, water limitation (loss of turgor) induces a more pendant leaf angle, but no change in leaf lamina. This relationship between petiole and lamina during water limitation is true for leaves of both tropical and temperate species of *Rhododendron* (Nilsen et al 1992). Idioblast abundance and leaf thickness is not a factor retaining leaf rigidity because leaves of temperate *Rhododendron* species, which have no idioblasts and tend to be thinner, are rigid as well. Therefore, leaf thickness is most related to leaf succulence not leaf rigidity. Variation in the relationship between leaf thickness and leaf succulence occurred because of both idioblast expression and mesophyll density.

There was significant five-fold variation (4 - 20%) in idioblast expression among leaves. That variation in expression was primarily due to increase in numbers rather than increase in size of idioblasts. The individual idioblast cell size determined was an underestimate because in any one thin section not all idioblast would be sectioned through their mid-axis. Thus, many of the idioblasts in an image were tangentially

sectioned which results in an underestimate of idioblast cell size. However, the total idioblast area in a section does represent the total volume of idioblasts in the leaf lamina volume. The structure of idioblasts was similar to that of idioblasts in other taxonomic groups such as those in plants in the Scrophulariaceae (Lersten and Curtis 1997).

A proportional reduction in idioblast expression as leaf thickness increased could simply be due to a constant total volume of idioblasts cells as leaf thickness changed. However, the negative relation between idioblast expression and leaf thickness in this study also was due to a decrease in the absolute total area of idioblasts ($p < 0.0001$) as leaf lamina thickness increased. Therefore, there was a biologically significant decrease in idioblast volume as leaves became thicker. Also, narrower longer leaves had higher idioblast expression in this study. This relationship suggests that idioblasts may function to ameliorate the effects of water limitation or high radiation because narrower and smaller leaves are characteristic of *Rhododendron* plants that inhabit higher light or drier environments (Nilsen and Bao 1990, Argent 2015). Also, the thickest leaves (most succulent) tended to be larger more lanceolate leaves. Consequently, the relationship between leaf form and idioblast expression may actually be due to the negative relationship between leaf thickness and idioblast expression. Thus, other attributes of leaf anatomy that are correlated with small leaves and narrow leaves are likely to be the primary factors determining the observed relationship between leaf form and idioblast expression.

4.42 Addressing hypothesis one—There was significant variation in idioblast expression among species represented by accessions used in this study. The data suggest that plants growing in different habitats have evolved differences in idioblast expression

that may relate to the significance of idioblasts to bulk leaf physiological traits.

Anatomical studies of idioblasts have traditionally focused on the anatomical structure and contents of the idioblasts rather than on the relative volume of idioblasts in relation to leaf lamina volume (Lersten and Curtis 1995, 2001, Cotta Coutinho et al. 2013, Matos et al. 2013, de Luna et al. 2014). Leaves of the sampled accessions in this study had no variation in idioblast anatomy (except size). All idioblasts in this study had large central vacuoles filled with aqueous solutions that stained positively with safranin O. The soluble contents and the concentrations of those contents could not be determined by this study, but due to their staining pattern it is likely that there are phenolics and tannins in the central vacuole aqueous solution (de Barros and Teixeira 2014). The presence of phenolic containing compounds suggests that the idioblasts may have a function for herbivore defense in tropical *Rhododendron*. However, leaves of temperate *Rhododendron* species contain a high content of phenolics as well (Qiang et al. 2011, Fu et al. 2012, Li et al. 2012) yet they do not have any idioblasts. Therefore, the idioblasts in tropical *Rhododendron* species likely have other functions besides herbivore defense that are related to the tropical environment.

4.43 Addressing hypothesis two—The second hypothesis posed in this study predicted that idioblast expression would increase in accordance with an increase in leaf succulence among plants. The results of this analysis do not support this hypothesis when the entire data set from the water relations experiment is used. In fact, succulence (g water cm⁻² leaf area) determined during pressure volume curves was most correlated with leaf thickness determined during anatomical analyses. This highly significant relationship between leaf thickness and leaf succulence is robust because the data were attained by

separate analytical procedures that minimize autocorrelation. Leaf thickness (and leaf succulence) was primarily determined by the proportion of leaf volume occupied by mesophyll (% mesophyll) not leaf idioblast expression. However, if only leaves less than 0.5 mm thick (50% of sampled plants) were used for the analysis, then there was a positive marginally significant association between idioblast expression and leaf succulence. Therefore, idioblast expression tends to be a determinant of leaf succulence for thinner leaves. We are unaware of any other studies that quantify the relationship between idioblast abundance and leaf thickness or succulence.

4.44 Addressing hypothesis three—Hypothesis three stated that stomatal pore index, a proxy for maximum stomatal conductance, would increase in accordance with an increase in idioblast expression among plants. This positive correlation would be expected if idioblasts serve as a water resource that buffers changes in water potential as the leaf loses water by transpiration. However, our data do not support this hypothesis. Instead, stomatal pore index was mostly associated with the succulence, which means that as leaves become more succulent they are able to have higher leaf transpiration rates. The inverse relationship between idioblast expression and leaf succulence traits resulted in a non-significant relationship between idioblast expression and stomatal pore index (SPI). SPI tends to be higher in high light environments and locations with low CO₂ concentration (high elevation). Plants in high light environments and high elevation sites tend to have relatively smaller leaf area and relatively thicker leaves than leaves on plants in low light or low elevation environments. The results for this research are contradictory to this general theory because accessions with larger leaves were generally thicker and had higher stomatal pore indices than accessions with smaller leaves. Many species of

Rhododendron in subgenus *Vireya* are epiphytic, which can interfere with the general theory for terrestrial plants because of the complexity of irradiance and water availability in the epiphytic environment. More needs to be known about the natural habitat of these species before the functional significance of leaf thickness and area can be interpreted.

4.45 Addressing hypothesis four—Hypothesis four posed that leaf capacitance, pre turgor-loss, would increase in relation to an increase in leaf idioblast expression among plants. However, idioblast expression was significantly correlated with WD at TLP and the difference between OP at 100% and OP at TLP, but not with capacitance pre- or post-TLP. Osmotic relationships were similar across species resulting in a similar slope for the line defining osmotic relationships in moisture release curves (data not presented). Thus, the slope of $1 / \text{water potential vs. WD}$ in the pre-TLP range tended to be steeper for thicker leaves with fewer idioblasts. This result indicates that the bulk modulus of elasticity for more succulent leaves was higher (stiffer cell walls) than thinner leaves with higher idioblast expression. In sum, thinner leaves with their higher idioblast expression were able to maintain turgor potential to more negative leaf water potentials because of the lower leaf lamina bulk modulus of elasticity. Therefore, high idioblast expression is significant for maintaining turgor to higher leaf water deficits without any effect on osmotic potential or leaf capacitance.

4.46 Summary and conclusion—Leaf succulence (leaf thickness) dominates the water relations traits of tropical *Rhododendron* leaves in subgenus *Vireya*. The effects of succulence are primarily related to bulk modulus of elasticity causing thick succulent leaves to lose turgor at a relatively positive leaf water potential and a relatively small water deficit. However, the relative abundance of idioblasts does have an important

relationship with leaf capacitance and WD at the TLP for thinner leaves. Both the WD at the TLP and capacitance increased as idioblast expression increased for relatively thin leaves. Thus, species of *Rhododendron* in subgenus *Vireya* with relatively small and thin leaves have idioblasts that likely function as a water buffering system.

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4.6 Tables

Table 4.1. Results from one-way ANOVA of species effect on morphological and anatomical traits of leaves from tropical *Rhododendron* species in subgenus *Vireya* growing under greenhouse conditions. Idioblast expression = % of the leaf lamina area occupied by idioblast cells, Stomatal pore index (SPI) = stomatal density (mean stomatal pore length²). Significant *p*-values (< 0.05) are presented in bold type. SS = Sum of Squares. Degrees of freedom = 16 in all cases.

Trait	Units	SS	F	Sig.
Leaf area	cm ²	9679.32	23.15	<0.0001
Leaf length	cm	210.75	25.24	<0.0001
Leaf L/W ratio	#	9.93	18.46	<0.0001
Leaf thickness	mm	0.66	15.14	<0.0001
Leaf succulence	g water / cm ²	0.0031	18.08	<0.0001
% mesophyll	% of thickness	1985.56	5.13	<0.0001
Idioblast density	mm ⁻²	6244.97	8.04	<0.0001
Idioblast area	mm ²	0.00002	6.33	<0.0001
Idioblast expression	% of leaf area	863.89	32.11	<0.0001
Stomatal density	mm ⁻²	338924	3.89	0.0006
Stomatal pore length	mm	0.002	3.46	0.0016
Stomatal pore index	#	0.3863	2.15	0.0338

Table 4.2. Results from one-way ANOVA of species effect on leaf water relations traits derived from moisture release curves of leaves from tropical *Rhododendron* species in subgenus *Vireya* growing under greenhouse conditions. WD = water deficit of bulk leaf tissue, OP = osmotic potential of bulk leaf tissue. Significant *p*-values (< 0.05) are presented in bold type. SS = Sum of Squares. Degrees of freedom = 16 in all cases.

Traits	Units	SS	F	Sig.
Capacitance pre-turgor loss	Mol m ⁻² MPa ⁻¹	8.142	3.698	0.0006
Capacitance post-turgor loss	Mol m ⁻² MPa ⁻¹	100.597	3.696	0.0007
WD at the turgor loss point	%	187.820	3.807	0.0005
OP at full turgor	MPa	1.439	3.670	0.0007
OP at turgor loss point	MPa	1.709	3.311	0.0016
OP full – OP turgor loss point	MPa	0.2459	1.3907	0.1955

Table 4.3. All the significant correlations among anatomical, stomatal, and water relations traits of leaves from tropical *Rhododendron* species in subgenus *Vireya*.

Idioblast density = number of idioblasts per leaf lamina length, Idioblast expression = % of the leaf lamina area occupied by idioblast cells, Leaf L/W ratio = leaf length / width, % mesophyll = the % of the leaf thickness occupied by spongy mesophyll tissue, WD = water deficit of bulk leaf tissue, OP = osmotic potential of bulk leaf tissue, TLP = turgor loss point of bulk leaf tissue, 100% = 100% saturated with water, SPI = stomatal pore index

Trait	Trait	Correlation Coefficient	Sig.
Idioblast density	Leaf L/W	0.8842	<0.0001
Idioblast density	Leaf thickness	-0.5524	<0.0001
Idioblast density	Leaf succulence	-0.6793	<0.0001
Idioblast density	Leaf % mesophyll	-0.3925	0.0025
Idioblast expression	Leaf L/W	0.6338	<0.0001
Idioblast expression	Leaf thickness	-0.4735	0.0001
Idioblast expression	Leaf succulence	-0.5055	<0.0001
Idioblast expression	Leaf % mesophyll	-0.6162	<0.0001
Stomatal density	Idioblast area	-0.3670	0.0039
Stomatal density	Idioblast expression	-0.3765	0.0030
Stomatal density	Leaf area	-0.3442	0.0087
Stomatal density	Leaf length	-0.3615	0.0057

Stomatal pore length	Leaf thickness	0.1368	0.0029
Stomatal pore length	Leaf succulence	0.0978	0.0077
Stomatal pore index	Leaf thickness	0.4061	0.0013
Stomatal pore index	Leaf succulence	0.3476	0.0081
Capacitance pre-TLP	Leaf area	-0.3875	0.0041
Capacitance pre-TLP	Leaf length	-0.2962	0.0313
Capacitance pre-TLP	Leaf L/W	0.2789	0.0453
Capacitance pre-TLP	Leaf thickness	0.4587	0.0006
Capacitance pre-TLP	Leaf succulence	0.5066	0.0001
Capacitance pre-TLP	Leaf % mesophyll	0.3248	0.0188
Capacitance post-TLP	Leaf L/W	-0.3129	0.0239
Capacitance post-TLP	Leaf thickness	0.3364	0.0147
Capacitance post-TLP	Leaf succulence	0.4071	0.0025
Capacitance post-TLP	Leaf % mesophyll	0.5197	<0.0001
Capacitance post-TLP	Idioblast density	0.3362	0.0148
Capacitance post-TLP	Idioblast expression	0.4120	0.0024
Capacitance post-TLP	Stomatal pore index	0.2848	0.0407
WD at TLP	Leaf area	-0.3135	0.0166
WD at TLP	Leaf length	-0.3046	0.0201
WD at TLP	Leaf L/W	0.2613	0.0496
WD at TLP	Idioblast density	0.3480	0.0080
WD at TLP	Idioblast expression	0.3326	0.0115
WD at TLP	Capacitance pre-turgor	0.3401	0.0127

WD at TLP	Capacitance post-turgor	0.2822	0.0407
OP at 100%	Leaf area	0.3332	0.0113
OP at 100%	Leaf length	0.2700	0.0422
OP at 100%	Leaf L/W	-0.4479	0.0005
OP at 100%	Capacitance pre-turgor	-0.3270	0.0180
OP at 100%	Capacitance post-turgor	0.5250	<0.0001
OP at TLP	Leaf area	0.2834	0.0326
OP at TLP	Leaf L/W	-0.3566	0.0065
OP at TLP	Capacitance pre-turgor	-0.3105	0.0251
OP at 100% – OP TLP	Leaf % mesophyll	-0.3185	0.0157
OP at 100% – OP TLP	Idioblast density	0.3009	0.0229
OP at 100% – OP TLP	Capacitance post-turgor	-0.5521	<0.0001

4.7 Figure legends

Figure 4.1. Representative cross section images of leaf lamina for several tropical *Rhododendron* species. Images A – D were stained with safranin O and fast green. Images were taken at 10 x objective lens, except image D and F were taken at 4 x objective lens. (A) *R. bryophyllum*, (B) *R. celebicum*, (C) *R. goodenoughii*, (D) *R. yongii*, (E) Unstained cross section of leaf lamina for *R. brookeanum*, (F) Paradermal section of leaf lamina for *R. zoelleri*.

Figure 4.2 (A) Regression of capacitance pre-TLP against idioblast expression for plants with leaves less than 0.5 mm thick. (B) Regression of capacitance post-TLP against idioblast expression. (C) Regression of WD at turgor loss point by idioblast expression as dependent variable.

4.8 Figures

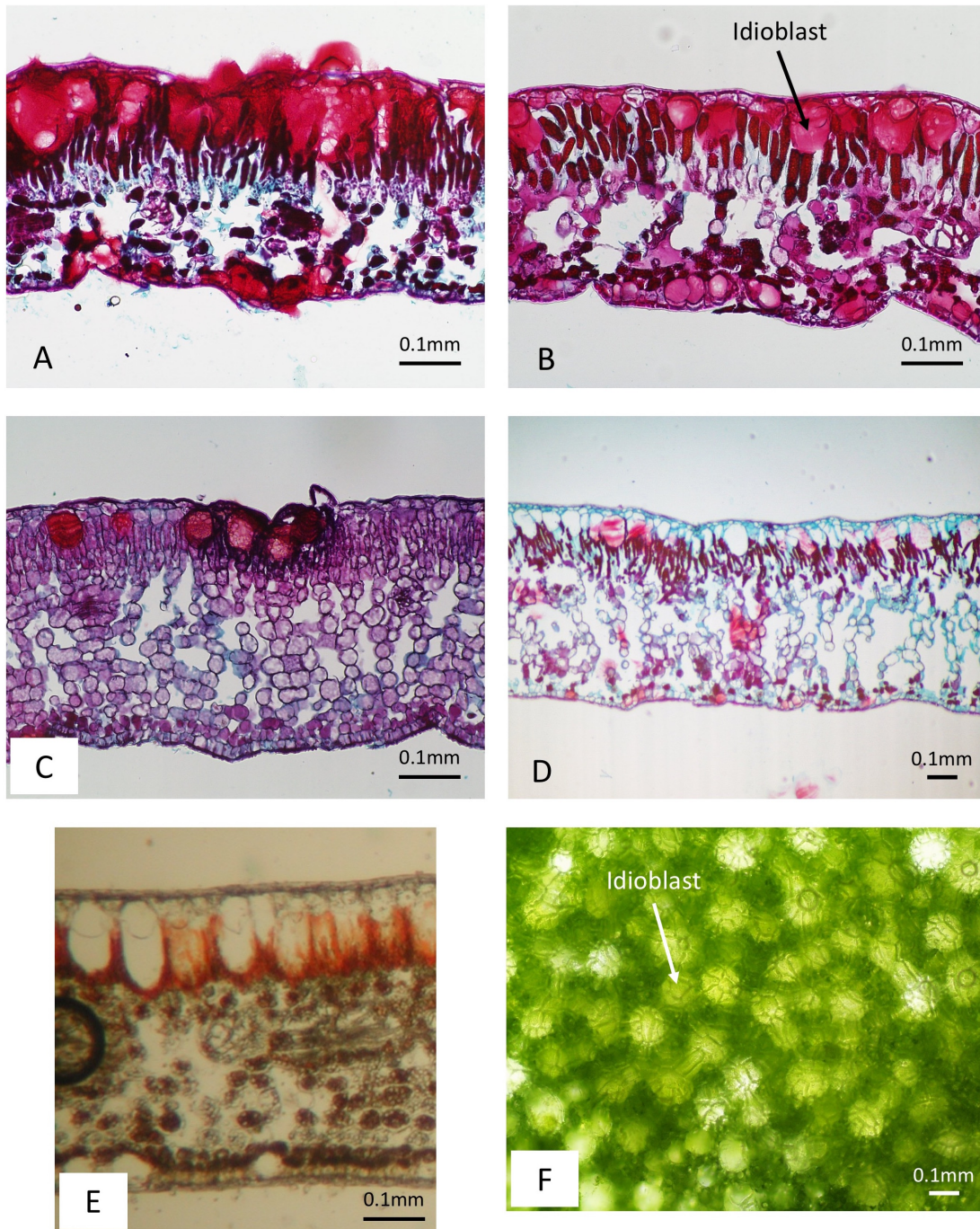


Figure 4.1. Representative cross section images of leaf lamina

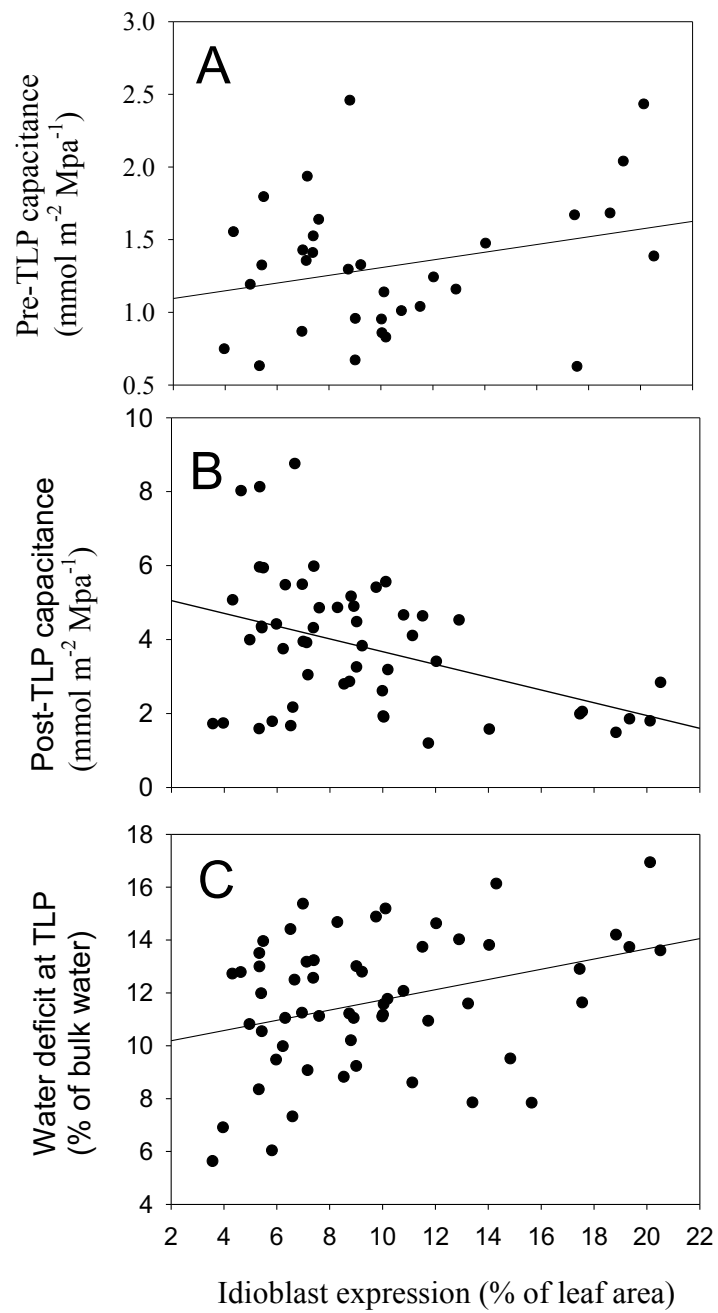


Figure 4.2. Regressions of water relations traits against idioblast expression

CHAPTER 5

Synthesis of research results: ecological implications of anatomical traits

5.1 Reflections and relationships with the Leaf Economic Spectrum

The Leaf Economic Spectrum (LES), as defined by Reiche (2014) explains that there are physiochemical constraints on the relationship between leaf functional traits in different habitats. Thin large leaves with relatively low density are commonly found on plants inhabiting high resource habitats and there is a continuum to smaller, denser leaves in habitats with low resource availability. Moreover, leaf longevity increases from leaves on species in high resource habitats to those on species in low resource habitats. These functional type constraints were formulated by examining leaf traits across the globe and are presented using a log-log plot. At any point across the global relationship there may be a lot of variation that is minimized by the log-log plot. This study examined the variation in stem and leaf anatomical traits in one group of plants that would reside far to the right of the LES. Evergreen *Rhododendron* shrubs are characterized by thick dense leaves that have extensive longevity, relatively low nitrogen concentration and relatively low photosynthetic rates (Nilsen 1986).

All evergreen *Rhododendron* taxa have entire leaves that are usually lanceolate (leaf length / leaf width (L/W) ratio of 2.5). The leaf area may vary significantly among species (Nilsen and Tulyanon 2015), but the shape and placement on the branch (whorled) is consistent. Although leaf longevity may vary based on light intensity (Nilsen 1986), leaves of evergreen *Rhododendron* species have relatively long life span. These characteristics of evergreen *Rhododendron* leaves support the placement of evergreen

Rhododendron species on the far right of the LES. Even though this group is placed at one extreme of the LES, there was a lot of variation in anatomical traits. If that variation reflects plant adaptation to habitat variation, then the LES misses many important adaptations related to leaf anatomy. Therefore, this research makes it clear that to fully understand how plant functional traits are associated with habitat variation research needs to go much further than just relying on the LES.

5.2 Limitations to productivity

There has been much consideration of the logical trade-off between hydraulic safety and hydraulic efficiency. In general, hydraulic safety refers to avoidance or repair of hydraulic dysfunction caused by embolisms due to drought or freeze-thaw cycles (Meinzer 2010). In contrast, hydraulic efficiency refers to the capacity to move water from roots to leaves. There is an inherent trade-off between these two processes because they both depend upon the anatomy of vessels. Small vessels favor safety while large vessels favor efficiency. This inherent trade-off is modified by vessel arrangement (ring porous vs. diffuse porous), vessel density, vessel contact (vessel network), as well as other factors. Therefore, the trade-off between safety and efficiency is not necessarily true in all cases (McCulloh 2010). There is some evidence that even closely related species in extreme environments can have evidence of this fundamental trade off (Medeiros and Pockman 2014). The data collected in this study were in agreement with Medeiros and Pockman (2014) because there was some evidence for the trade-off between safety and efficiency between species from temperate and tropical environments.

However, the difference in safety was relatively small and both the temperate and tropical species wood should be classified as highly safe.

The strong safety characteristic of the *Rhododendron* wood was a result of relatively small diameter vessels. Consequently, the theoretical specific conductivity also was found to be relatively small compared to many other species in the tropics and temperate zones. The highly safe vascular system of *Rhododendron* species portends water flow limitation during periods of higher evaporative demand. The result of the constraint in water flow would be low leaf water potential. However, studies of both the tropical species in this study and temperate species in other studies (Nilsen 1987, Lipp and Nilsen 1997) show that complete turgor loss (stomatal closure) occurs at a relatively high water potential and small water deficit. Therefore, the vascular structure of *Rhododendron* species (in both temperate and tropical habitats) restricts water flow, reduces transpiration and results in low photosynthesis culminating in relatively slow growth rate. All anatomical traits and water relations measurements collected in this study point to the vascular system limitation of potential productivity in evergreen *Rhododendron* species.

5.3 Consequences to habitat tolerances

Rhododendron originated in the circumboreal forest of Laurasia during a time of relative high humidity, warm temperature and higher carbon dioxide concentration (Milne. and Abbott 2002). Since its origin *Rhododendron* has diversified into colder climates on temperate mountains and into warmer climates in the tropics. Or, continental movements have created novel climates to which local *Rhododendron* species had to

acclimate, some of which became relict species of a broader distribution (Milne 2004). In both cases, innovation was needed for adaptation to the new extreme climatic conditions. Adaptation to temperate regions, where temperature changes dramatically over the season, required new tolerance of freeze-thaw cycles. It is possible that the original *Rhododendron* species had narrow vessels and were pre-adapted to tolerating the freeze-thaw cycles of the temperate mountain regions. This research indicated that leaf innovation may have been multi layered epidermis (hypodermis) to avoid high ultraviolet radiation in the open forest of mountain regions. Another important innovation in leaves was thermonastic leaf movement (Nilsen 1992), which protected leaves from radiation damage during cold periods of the year.

Diversification into the tropics is a different problem because innovation against freeze-thaw is unnecessary and potentially maladaptive. This study showed that the vascular system of tropical evergreen *Rhododendron* shrubs had higher efficiency than that of temperate *Rhododendron* shrubs. This result suggests that increasing vascular efficiency was associated with the diversification into the tropics. However, the enhancement of efficiency was relatively small. Therefore, the vascular system of tropical *Rhododendron* species is still classified as a “safe” wood. The constraints placed on water flow for the tropical *Rhododendron* species may lead to significant water stress because of the potential high evaporative demand in some tropical environments. In particular, epiphytic habitats and those in open disturbed areas. Many tropical *Rhododendron* species inhabit these regions of relatively high radiation and low water storage (Argent 2015). Therefore, leaf innovations for managing water balance were needed during the diversification of *Rhododendron* species into the tropics because

vascular efficiency did not change much. Succulence was found to be much more abundant in leaves of tropical *Rhododendron* species. Succulence is a well-known method of maintaining water balance during periods of water limitation. A strong innovation in leaves of tropical species was idioblasts. This study found that idioblasts do influence leaf water balance (increasing capacitance) for leaves that are not already succulent (thin leaves). The appearance of idioblasts in all the leaves of tropical *Rhododendron* could have been because the lineage that diversified into the tropics happened to have idioblasts, or that idioblasts were an important leaf innovation that allowed *Rhododendron* to diversify into the tropics. It is more likely that idioblasts were important for diversification into the tropics because they have never been lost during the diversification throughout the tropics.

5.4 Limitations and potential improvement

This type of survey of anatomical traits broadly across a genus has limitations. Primary among those limitations is the species selection. *Rhododendron* has approximately 1,000 species, 350 of which are tropical. It would not be possible to survey all the known species in this group; therefore, a subsample needs to be made. A survey uses one subsample (in this study 80 species), which could be a biased subsample. The bias may result in finding differences that do not exist or missing differences that do exist. A fully comprehensive survey would include all species or multiple random subsamples. Neither of these options are feasible based on a graduate program.

Another limitation is the ability to obtain samples from a single source. The samples used in this survey were from the Royal Botanic Garden Edinburgh, UK, the

Rhododendron Species Foundation and Botanical Garden in Federal Way, WA, a common garden in Volcano Village, HI and Biological Sciences-VBI Plant Growth Facility (VT-PGF) in Blacksburg, VA. Using accessions from one reputable source may limit the variation and improve the resolution of the habitat effects. However, no one source can supply enough species in each of the habitat types.

The sample selected for this study was a random selection of accessions in each habitat type (tropical and temperate). At the beginning of this project, an attempt was made to collect accessions that represent all the clades resolved in the recent phylogenetic studies. It was impossible to locate enough of the species used in that publication to study the phylogenetic basis of anatomical variation. This study would have been improved if it was possible to obtain enough species to strengthen the phylogenetic signal and utilize the independent contrasts method to analyze if certain anatomical traits were determined by phylogeny or environment.

All surveys are necessarily correlative studies. Correlation, regression and ANOVA were used in this study to understand the relationships between habitat or elevation and anatomical traits. This study cannot assign cause and effect relationships between habitat and anatomical trait. Improvements on the cause and effect relationships might be accomplished by studies of hybrids between temperate and tropical species or by locating mutants of a species that lacks a particular anatomical innovation.

Perhaps this study could be improved by collecting all samples from plants in their native ranges. Traveling to all the native ranges would be extremely difficult because many species occur in remote locations. It may be possible to select a smaller sample size of species that are accessible in the field and compare the field traits to those

of plants growing in the common gardens or greenhouses. Comparing the anatomical traits of the field sample to the sample from plants in the common garden may help to understand the common garden effect on anatomical traits. A comparison between greenhouse and field-grown plant was done in this study. Most of the traits were not significantly different between greenhouse and field-grown (Table 5.1). However, % mesophyll was significantly lower in greenhouse growing plants. Because of this, the difference between temperate and tropical might have been undetected, because field-grown tropical plants would have had higher % mesophyll. In contrast, A_{mes}/A ratio and stomatal pore index were found to be significantly higher in greenhouse-grown plants compared to the field-grown plants. This result suggested the differences in A_{mes}/A ratio and stomatal pore index found between temperate and tropical may be a greenhouse artifact. However, the test of greenhouse effect is based on very small sample size.

Other anatomical traits might be added to the list used in this study. The study would be improved if physiological traits such as hydraulic conductance, nitrogen (N) concentration or photosynthesis were included in the study. Perhaps the addition of the physiological traits would increase the linkage of this study with the LES. Roots are the foundation of water relation traits because all water passes through roots to the stems and leaves. Adding anatomical or physiological traits of roots may increase the strength of this study as well.

5.5 General conclusions from this study

The LES is a good base for understanding fundamental plant characteristics, but a lot of variation in adaptive traits is hidden within each functional group of the LES.

Evergreen *Rhododendron* shrubs are classified at one extreme of the LES, yet they inhabit many different habitats from arctic fell fields to lowland wet tropical forest. The variation in anatomical traits found in this study is one factor that allows evergreen *Rhododendron* species to colonize different habitat types.

Vascular tissues of evergreen *Rhododendron* species are inherently “safe”, which puts a constraint on water flow. The constraint on water flow restricts evergreen *Rhododendron* species to low productivity, yet provides adaptation to temperate climates. The constraint on water flow in tropical evergreen *Rhododendron* species stem has led to the development of water management innovations such as succulence and idioblasts. Moreover, low productivity likely makes *Rhododendron* weakly competitive with other faster growing tropical species and restricts the *Rhododendron* species to marginal habitats such as disturbed areas and epiphytic locations. Examining the variation in anatomical traits among evergreen *Rhododendron* species lead to insight about traits that constrain plant performance and traits that determine habitat preference.

5.6 Literature cited

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5.7 Table

Table 5.1. F-test table to compare the significant effect of growing condition between greenhouse-grown (VT-PGF, Blacksburg, VA) and field-grown (Volcano Village, HI) leaf traits.

Leaf Traits	Sig.
Leaf Area	0.631
Leaf L/W Ratio	0.602
Leaf Thickness	0.127
% Mesophyll	0.053*
Mesophyll Density	0.010**
Idioblast Expression	0.793
Idioblast Density	0.918
Stomatal Density	0.140
Stomatal Pore Length	0.893
Stomatal Pore Index	0.055*