MECHANISTIC BASIS OF GAS EXCHANGE IN THE TERRESTRIAL ASTOMATAL ACID METABOLISM PLANT *Chiloschista lunifera* (REICHB.F.) J. J. SM. (ORCHIDACEAE).

By

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Crassulacean acid metabolism (CAM) represents a versatile and highly plastic photosynthetic pathway, showing a great degree of variability in biochemistry, expression and anatomy between various CAM species. While a large body of literature exists addressing biochemical and expressional variation in CAM, relatively little work has been done addressing the anatomical variants of CAM, particularly Terrestrial Astomatal Acid Metabolism (TAAM) and its occurrence in the photosynthetic roots of orchids.

While these TAAM roots lack stomata, they do possess specialized aeration complexes embedded in the root surface which are thought to provide a diffusive path for gas exchange. It has been proposed that a pair of differentially thickened cells within this structure may act similarly to stomatal guard cells, serving to regulate gas exchange. However, the few studies that have been conducted to date have focused solely on carbon

and as a result have failed to provide strong evidence for or against a regulatory mechanism.

Diel gas exchange patterns for *Chiloschista lunifera* were indicative of CAM. Transpiration rates tracked those of carbon assimilation, with maximum rates of both occurring at night. Measurements of water vapor flux across isolated velamen indicated an ability of velamen to absorb water vapor at a vapor pressure deficit (VPD) less than 2 kPa, and likely played a role in the steady decrease observed in transpiration rates with decreasing VPD. The consistent diurnal variation in transpiration and the rapid decrease in transpiration rates in response to high VPD imply an active means of regulating gas exchange in *C. lunifera*. Further study is necessary to determine if the regulatory mechanism is based on changes in cortical component aperture, or is the result of some other mechanism.

CHAPTER 1
INTRODUCTION

Crassulacean acid metabolism (CAM) represents a versatile and highly plastic photosynthetic pathway, allowing for maximum carbon acquisition under carbon limited conditions (Cockburn 1985, Cushman 2001, Lüttge 2004). Under the traditional view of CAM, carbon uptake is dominated by nocturnal fixation of CO_2 by phosphoenyol pyruvate carboxylase (PEPC) to produce malic acid, which is stored in the vacuole. Malate is then remobilized behind closed stomata and decarboxylated in the light providing CO_2 for Rubisco to act upon (Cockburn 1985, Lüttge 1987, Osmond 1978). However, because of the great deal of taxonomic diversity among CAM plants (e.g. 16 000 species from 33 families [Cushman 2001, Cushman and Borland 2002, Zotz 2002]) and is undoubtedly polyphyletic origins a vast array of CAM variants exist (Cushman 2001), making a comprehensive and coherent definition of CAM difficult (Lüttge 2004).

The wealth of variation among CAM plants results from deviations in biochemistry, expression and anatomy from Osmond's (1978) classical description of CAM. While a great deal of work has been done regarding CAM variation resulting from deviations in biochemistry (e.g., Bakrim *et al.* 2001, Chen and Nose 2004, Cockburn 1985, Cushman and Bonhert 1999, Drincovich *et al.* 2001, Lüttge 2000, Taybi *et al.* 2004) and expression (e.g., Borland and Grifiths 1990, Cockburn 1985, Cockburn 1998,

Cushman 2001, Cushman and Bonhert 1999, Cushman and Borland 2002, Kluge *et al.* 1997, Lüttge 1987, Lüttge 2000, Zotz 2002), almost no work has been done addressing the anatomical variants of CAM, particularly Terrestrial Astomotal Acid Metabolism (TAAM).

Terrestrial Astomotal Acid Metabolism (TAAM)

Terrestrial Astomotal Acid Metabolism is only known from two phylogeneticly distinct groups of plants, the rare member of the Isoetaceae, *Isoetes andicola* (= *Stylites andicola*) and the photosynthetic velamentous roots of orchids. In both cases stomata are absent from the photosynthetic organs and carbon fixation is dominated by PEPC (Cockburn 1985, 1998).

In *Isoetes andicola* carbon is taken up via an extensive root system from a moist CO_2-rich substratum. The photosynthetic organs are covered by a thick waxy cuticle lacking stomata, preventing direct gas exchange with the atmosphere (Cockburn 1985, 1998). While in general the CAM pathway and an absence of stomata are not unusual in *Isoetes*, they are for terrestrial members of the genus (i.e. stomata are absent in aquatic species), suggesting *I. andicola* as an intermediate between terrestrial and aquatic forms (Cockburn 1985).

The situation for TAAM in the photosynthetic velamentous roots of orchids is quite different then in *I. andicola*. In both cases roots serve as the primary organ of carbon acquisition by TAAM (Cockburn 1985, 1998), however the photosynthetic velamentous roots of orchids exists exposed to the atmosphere in an environment with sporadically available water resources (Benzing and Ott 1981). These roots may be exposed to long periods of drought between rain events, and therefore must have some

means of regulating transpirational losses to the environment (Benzing and Ott 1981, Benzing *et al.* 1983) while allowing CO_2 to diffuse in from the surrounding atmosphere.

Vegetative Reduction and the Shootless Habit

The Orchidaceae is known for its unsurpassed degree of ecological diversity and numerous deviations from the typical body plans exhibited by vascular plants (Figure 1-1) (Benzing and Ott 1981). One of the most interesting deviations is the allocation of photosynthetic carbon acquisition to the roots and the subsequent loss of leaves and reduction of the stem exhibited by shootless taxa. While photosynthetic roots are present in many epiphytic orchids exhibiting a broad array of vegetative morphologies, they are most refined in the shootless species where they serve as the sole means of carbon gain (Benzing and Ott 1981, Benzing *et al.* 1983, Carlsward 2004).

The shootless habit is not common within the Orchidaceae (Benzing and Ott 1981) (comprising less than one percent of the family), and is represented in only the tribe Vandeae (Carlsward 2004, Carlsward *et al.* 2003). Within the Vandeae photosynthetic roots and the monopodial growth habit are common though and presumably provided the framework from which the shootless habit could arise (Carlsward 2004).

While the forces driving the evolution of shootlessness are not well understood, the most widely cited benefit is the suspected improved water economy associated with the habit. By reducing their surface area, these plants presumably decrease transpirational demand and increase water use efficiency (Benzing and Ott 1981, Benzing *et al.* 1983, Cockburn 1985, Rolfe 1914).

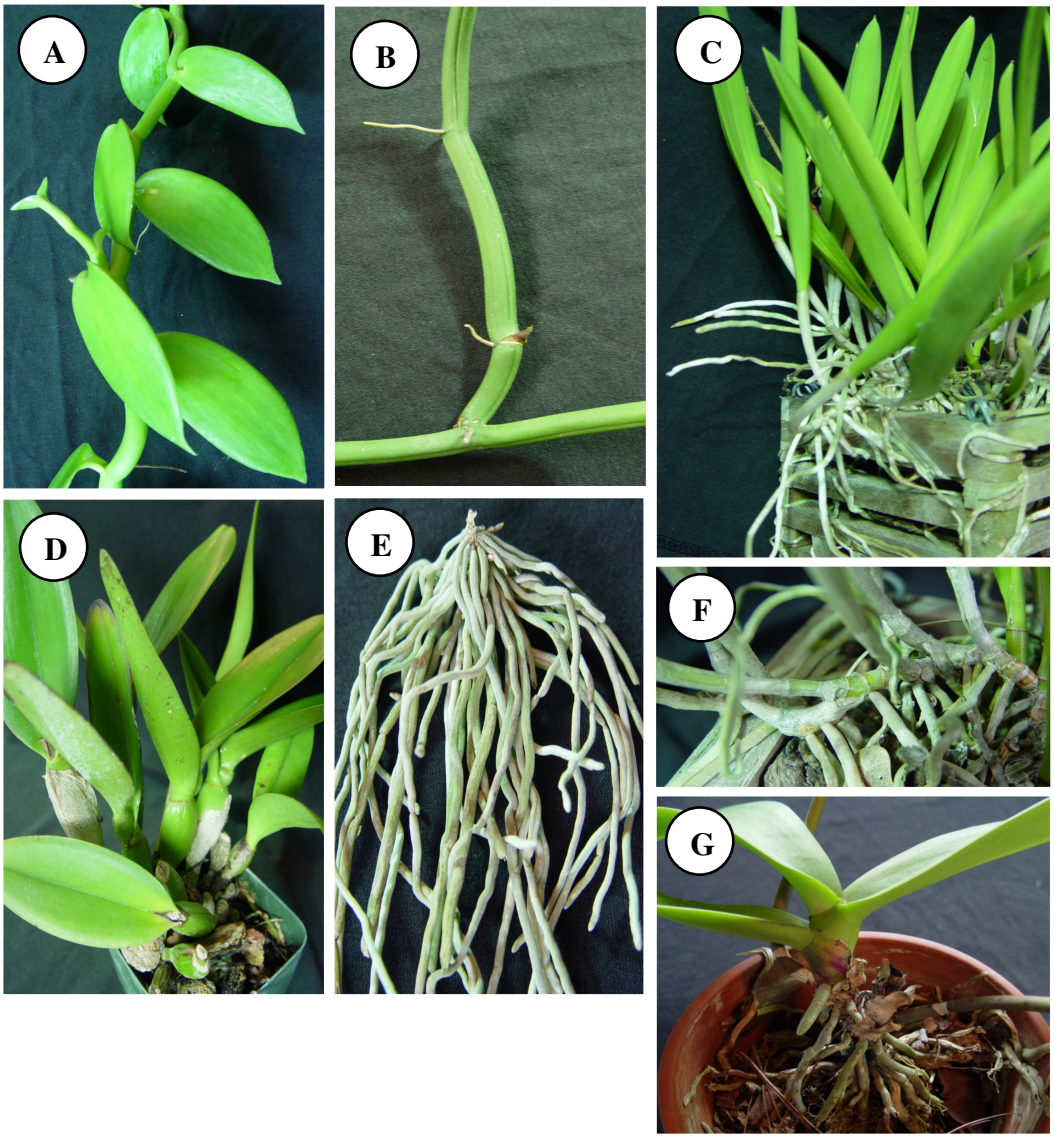

Figure 1-1: Vegetative morphologies of various orchids. A. *Vanilla planifolia* a leafy monopodial species which exhibits both photosynthetic stems and roots. B. *Vanilla barbellata* a leafless monopodial species reliant solely on photosynthetic stems. C. *Brassavola nodosa* a leafy sympodial species with stems and roots capable of photosynthesis. D. *Cattleya duisosa* x *Blc* "California girl" a leafy sympodial species with stems and roots capable of photosynthesis, and stems modified for water storage (psuedobulbs). E. *Chiloschista lunifera* a shootless species reliant solely on photosynthetic roots for carbon acquisition. F. Close up of *B. nodosa* showing sympodial growth habit. G. *Phalaenopsis* (unnamed hybrid) a leafy monopodial species with stems and roots capable of photosynthesis.

However, little physical evidence supports this theory. It has been shown that velamen thickness is positively correlated with habitat aridity (Benzing and Ott 1981, Sanford and Adanlawo 1973). One would expect that if these species had historically been exposed to such arid conditions to force the extreme vegetative reduction seen today, as suggested by some authors (Cockburn 1985, Rolfe 1914), shootless orchids would posses thick velamen indicative of species originating from arid habitats (Benzing and Ott 1981). However, shootless taxa tend to posses thin velamen, and in some species, such as *Campylocentrum pachyrrhizum* the velamen is almost completely eroded away on mature roots (Benzing *et al.* 1983). Detailed habitat information is scanty for most shootless species, but it is clear from their needs under cultivation that these species are intolerant of conditions where transpirational demands are high and ambient humidity is low (pers. obs.). Taken together this suggests that water economy is likely not a primary driving force behind the origin of shootlessness.

It has been suggested that the CAM based mode of photosynthesis exhibited by these roots is evidence that these plants had to historically coupe with habitat aridity (Benzing and Ott 1981). While CAM has traditionally been thought of as a means to increase carbon gain under water limited conditions (Cockburn 1985, Lüttge 1987, Osmond 1978), it is better thought of as a means to increase carbon gain under carbon limited conditions (Cockburn 1985, Cushman 2001, Lüttge 2004). Following this line of reasoning the occurrence of CAM in the photosynthetic roots of those leafy species that preceded the development of the shootless habit is quite reasonable. The elaborate ventilation systems seen in many shootless taxa are absent or highly under developed among leafy orchids (Benzing *et al.* 1983). This presumably poses a rather large

resistance to the diffusion of carbon dioxide in to these roots. CAM allows for maximized carbon gain under these conditions by allowing the plant to generate a much greater concentration gradient of CO_2 between the root cortex and the surrounding atmosphere, driving the influx of CO_2 in to the plant. In addition remobilization of CO_2 from malate during the light vastly increases the internal CO_2:O_2 ratio suppressing costly photorespiration due to the oxgenase activity of Rubisco (Keeley and Rundel 2003).

An argument can also be made for a non-carbon acquisition based origin for CAM in the photosynthetic roots of orchids. CAM allows for very high solute (generally malate) concentrations to be generated, resulting in a large osmotic potential that can drive the influx of water into the plant (Lüttge 1987). As rain events are sporadic and short lived in the epiphytic biotope (Benzing and Ott 1981), the amount of water that can be scavenged during any given wetting event is of key importance to the health of the plant. Water uptake by orchid roots is driven by two factors, water movement into the velamen by capillary action and subsequent movement into the root cortex across an osmotic gradient (Benzing and Ott 1981, Benzing *et al.* 1983). As velamen is composed of non-living cells it is likely that the plant can not exert any direct control over the rate at which water is pulled in to the velamen. However, by increasing the solute concentration inside the root, the rate of osmoticly driven uptake from the velamen to the cortex could be greatly increased. Potentially reducing the residence time for water in the velamen and increasing the amount of water that can be pulled into the root following a wetting event. While this would suggest a water economy based origin for CAM in the photosynthetic root, it doesn't necessitate a water limitation based origin for shootlessness.

An epiparastic mode of nutrition has also been suggested as a driving force behind the vegetative reduction seen in shootless taxa (Benzing and Ott 1981, Johansson 1977, Ruinen 1953). However, under cultivation these species do best when grown on non-decomposable substrates such as coarse metal or plastic mesh (Whitten pers. comm.), suggesting that nutrients scavenged directly from the host (if at all) are not essential for plant survival. Also the presence of epiphytotic relationships, a possible means of epiparastism, while not thought to be uncommon among orchids have as far as this author is aware have not been documented among shootless taxa.

The most recent supposition as to the forces driving the evolution of shootlessness is the suspected increased nutrient economy of this habit (Benzing and Ott 1981, Benzing *et al.* 1983). Nutrient inputs are highly limited in the epiphytic biotope. Occurring high in the forest canopy, epiphytes are cut off from the lavish nutrient pools available to terrestrially rooted vegetation, forcing them to rely on sporadic and short lived nutrient pulses associated with rain events and dust deposition (Nadkarni 1985). As a result they have developed many, often unusual, strategies to cope with this feast or famine situation, including litter impounding leaf configurations, phytotelmata, absorptive trichomes, insectivory, myrmecophytism, and the occurrence of mycorrhizae (Benzing 1990, Hietz *et al.* 2002, Nadkarni 1985, Nadkarni and Matelson 1991). It has been suggested that by not investing valuable nutrients in leaves and supporting structures where they would become permanently fixed, shootless orchids maybe able to reallocate these nutrients to increase fecundity (Benzing *et al.* 1983).

Photosynthetic Velamentous Roots

The roots of epiphytic orchids are covered in a spongy layer of hydrophilic cells called the velamen, which serves to take up water and mineral nutrients from the environment (Benzing and Ott 1981, Benzing *et al*. 1982, Benzing *et al*. 1983, Dycus and Knudson 1957, Hew *et al.* 1993, Pridgeon 1987). Upon a wetting event the velamen cells rapidly engorge with water and dissolved minerals, which are pulled into the root through highly cytoplasmic "passage cells" in the exodermis. These cells are embedded in a matrix of highly suberised "U" cells that provide a barrier to water movement. The passage cells contain an extensive membrane network and a greater density of mitochondria than surrounding cells, suggesting some active mechanism for transporting solutes into the root (Benzing *et al*. 1982, Benzing *et al*. 1983). The ability of these cells to move solutes and water into the root is contingent on the length of time that the adjoining velamina remain engorged with water (Benzing *et al*. 1982, Benzing *et al*. 1983), and may be one of the driving forces behind the correlation between velamen thickness and habitat aridity; As orchids with thicker velamen tend to be found in more arid habitats (Sanford and Adanlawo 1973). Presumably this thicker velamen would remain saturated longer and allow for greater nutrient and water uptake.

The tissue layer comprising the velamen is derived from periclinal divisions of dermatogen resulting in a multiple epidermis (Engard 1944). This layer maybe comprised of anywhere between one and twenty layers dependent on species (Pridgeon 1987, Porembski and Barthlott 1988). Velamen cells are non-living and air filled at maturity, which occurs within the first few millimeters behind the root tip (Pridgeon 1987). Walls of the velamen cells undergo deferential deposition of cellulose fibrils prior to cell death

resulting in secondary thickenings (Pridgeon 1987). These thickenings maybe helical, parallel or less often forked in nature (Noel 1974, Pridgeon 1987) and are thought to stabilize and support the relatively thin walls of velamen cells (Porembski and Barthlott 1988).

As a result of drying and shrinking of the cell walls of the velamen following cell death, pores develop ranging in size from 1 μm to 50 μm in diameter (Pridgeon 1987). These pores originate as small tears and elliptical slits in pit fields within the velamen cell walls, and rapidly elongate as the velamen shrinks (Noel 1974, Pridgeon 1987). The secondary wall thickenings supporting the velamen likely act to limit the size of large pores by preventing further elongation (Pridgeon 1987). Species with helical thickenings, most common in epiphytes (Stern and Carlsward 2004), generally exhibit the largest pores (Porembski and Barthlott 1988). Across all species the inner most tangential velamen wall shows the greatest density of pores, particularly above passage cells in the exodermis (Porembski and Barthlott 1988).

Composition of velamen cell walls is highly variable among different orchid taxa (Noel 1974, Pridgeon 1987). All wall layers are cellulosic in nature and exhibit widely varying degrees of lignin and suberin impregnation (Benzing *et al.* 1983, Pridgeon 1987). In multilayered velamen lignification is greatest in the walls of the inner most velamen layers and decreases in the outer layers (Pridgeon 1987, Sanford and Adanlawo 1973). In a study of velamen properties Giles and Agnihotri (1968) determined the velamen of *Vanda suavis* to be comprised of 51.5 % cellulose with a specific surface area of 425 $m^{2} \cdot g^{-1}$, an interesting finding as this is approximately twice that observed for wood and jute and greater than three times that for cotton.

Velamen structure also varies with taxon, but is relatively constant across a given species or in many cases a greater taxonomic grouping, particularly at the tribal and subtribal levels (Pirdgeon 1987, Porembski and Barthlott 1988). Porembski and Barthlott (1988) identified ten distinct, and one unspecified, velamen types in an exhaustive survey of 344 species from 262 genera, based on properties of the cell wall, number and stratification of the velamen layers, and tilosomes, in addition to other morphological characters of the root.

Velamen physiology as it relates to gas fluxes in and out of the root has been generally ignored since the discovery of velamen in the mid 1800s. Speculation as to velamen function, following in the first few decades after its discovery, often cited a role of velamen in condensing water vapor, though little or no experimental evidence supported this conclusion (see Pridgeon 1987 for review). As a result this view quickly fell out of favor and the vast majority of subsequent physiological research concerning velamen function has focused on its now well established role in water and nutrient uptake (see Pridgeon 1987 for review).

Velamen is often cited as means to resist desiccation under the arid conditions associated with epiphytism, either by increasing the boundary layer surrounding the root (Pridgeon 1987) or by some undocumented resistive property of the tissue. While velamen thickness has been shown to correlate with habitat aridity (Sanford and Adanlawo 1973), investigations into the desiccation resistance of various orchid species has not shown a similar relationship (Benzing *et al.* 1983). Benzing *et al.* (1983) found that among the ten orchid taxa studied *Polyradicion lindenii* (= *Dendrophylax lindenii* see Carlsward *et al.* 2003) showed the greatest resistance to desiccation, though this species

has only a moderate surface to volume ratio and thin velamen, two to four cells thick (Benzing *et al.* 1983, Carlsward 2004). Similarly structured roots of other species with thicker velamen layers desiccated much faster, suggesting that the velamen layer may not play a significant role in desiccation resistance (Benzing *et al.* 1983).

The author is aware of only one investigation in the modern literature[*] examining the ability of velamen to absorb water vapor. Giles and Agnihotri (1968) developed absorption isotherms for the velamen of both *Philodendron gigantum* (Aeraceae) and *Vanda suavis* (Orchidaceae). The velamen of both species, though from quite different taxonomic backgrounds, showed similar chemical composition and an amazing ability to absorb water vapor. *V. suavis* was able to absorb 15% of its dry weight at a relative humidity (RH) as low as 50% (at 20°C), and showed a maximum absorption of nearly 24% of its dry weight at 98% RH (at 20°C). These values are much greater than those observed for other cellulosic materials such as wood, jute and cotton (Giles and Agnihotri 1968).

Within the cortex of velamentous roots lie thin walled living parenchyma, which may contain chloroplast, and non-living tracheoidal idioblasts resembling water conducting cells (Carlsward 2004, Benzing *et al*. 1983, Solereder and Meyer 1930). The cell walls of these tracheoidal idioblasts lack suberin and lignin, making it possible for them function as collapsible water storage cells similar to those found in the parenchyma of many succulents. In addition the cortex of photosynthetic orchid roots also contain a network of gas filled passages, which approach or are connected to specialized aeration complexes spanning the exodermis and velamen (discussed below). Among leafy orchids these intercellular air spaces are within the size range for the intercellular air spaces

[*] Modern used here refers to publications post 1900.

found in the leaves of CAM and C_4 species. Shootless taxa possess larger air spaces, but they still remain smaller than those observed for C_3 plants (Benzing *et al.* 1983).

The velamen is especially well developed among shootless orchids, and possesses specialized aeration complexes which presumably provide a diffusive path for gas exchange (Benzing and Ott 1981, Benzing *et al*. 1983, Cockburn 1985, Cockburn *et al.* 1985). Though these aeration complexes are not restricted to shootless taxa, they are most refined in these species. Embedded within the velamen of these roots are areas of highly suberised cells, the pneumathode, which remain void even when the velamen is fully engorged with water (Benzing and Ott 1981, Benzing *et al*. 1983, Dycus & Knudson 1957, Pridgeon 1987). These regions often sit atop a thin walled aeration cell, which in turn sits atop two differentially thickened cortical components in the root's exodermis. These cortical components potentially act as guard cells and may provide a means of regulating gas exchange (Benzing and Ott 1981, Benzing *et al*. 1983).

Benzing *et al.* (1983) proposed two methods by which the aeration complex could regulate gas exchange. They suggested that (1) by expansion and contraction of the two cortical components, that they could function analogously to stomatal guard cells and could provide a homiohydrous mode of regulating gas exchange. Alternatively, (2) as the roots swells or contracts in relation to the overall root water status the two cortical components may be forced together or pulled apart, providing a poikilohydrous mode of regulation (Benzing *et al.* 1983). However, data from the few studies looking at gas-exchange in photosynthetic orchid roots has not provided strong evidence for or against an aeration complex based regulatory mechanism (Benzing *et al.* 1983, Cockburn *et al.* 1985).

It seems quite likely that some regulatory mechanism exists, though data supporting this are inconclusive. In their work with *P. lindenii* Benzing *et al.* (1983) demonstrated that this species showed the greatest resistance to desiccation and that this could not be attributed to properties of the velamen. Similarly structured roots of a leafy species with a thicker velamen layer desiccated nearly twice as fast, suggesting that *P. lindenii* had at least some control over water loss (Benzing *et al.* 1983). Whether or not this increased desiccation resistance is the result of the more refined aeration complexes found in the roots of *P. lindenii* is yet to be determined.

In their work with *Chiloschista usneoides* Cockburn *et al.* (1985), conclude that it was highly unlikely that the aeration complex could regulate gas exchange (Cockburn 1985). They suggested that the small loss of CO_2 observed during the light in *C. usneoides* was not the result of an increased diffusive resistance resulting from reduction in the cortical component aperture, but resulted from the maintenance of internal CO_2 concentration of the plant near atmospheric concentration (Cockburn *et al.* 1985). Samples taken from the intercellular air spaces of these roots by the authors were admittedly contaminated by air from outside the root, and may have been an inaccurate representation of actual internal CO_2 concentrations.

While no direct observation of cortical component aperture over any time course has been made to date (presumably because of obstruction by the overlying velamen layer), in her work with the anatomy of Vandeae, Carlsward (pers. comm.) noted a great deal of variation in the distance between the cortical components within a given species. The basis of this variation remains undetermined. But it seems likely that it could result

from changes in turgor during the process of preparing specimens for anatomical study and so maybe analogous to changes in turgor in the living root effecting cortical component aperture.

Objectives

Terrestrial Astomatal Acid Metabolism represents a unique and poorly understood anatomical variation on the tradition CAM pathway. While the occurrence of TAAM is limited to only two plant groups (Cockburn 1985), the diversity of CAM orchids in tropical forest may mean that TAAM is exhibited by as many as 75% of all CAM species in a particular area (Zotz and Ziegler 1997). Given that much of our understanding of CAM physiology stresses the importance of stomata in regulating gas exchange (Cockburn 1983, Cockburn 1985, Lüttge 1987), TAAM represents a potentially large gap in our understanding of CAM. It is difficult to imagine how a plant could maintain the associated high internal CO_2 concentrations of a CAM pathway, or how these plants could mediate water losses in the water limited environment of the forest canopy without some mechanism to regulate gas exchange. The principal goals of my research were to develop a model describing gas exchange for photosynthetic velamentous orchid roots exhibiting TAAM and to provide evidence either for or against an aeration complex based regulatory mechanism.

CHAPTER 2
A MODEL OF GAS EXCHANGE IN PHOTOSYNTHETIC VELAMENTOUS ROOTS

Introduction

The photosynthetic roots of epiphytic Orchids exhibit an unusual and poorly understood variation of Crassulacean Acid Metabolism (CAM). While thought to follow the same general biochemistry as other CAM variations, Terrestrial Astomatal Acid Metabolism (TAAM) is unique in that there is a total absence of stomata from the photosynthetic organs (Cockburn 1985). While TAAM is known to occur in only two phylogeneticly distinct plant groups, the rare member of the Isoetaceae *Isoetes andicola* and the photosynthetic roots of orchids (Cockburn 1985), its occurrence among tropical CAM species maybe quite common as orchids may comprise 75% of all CAM species in a given tropical forest (Zotz and Ziegler 1997).

Diel gas exchange patterns for the photosynthetic roots of orchids are generally indicative of CAM, showing predominate carbon assimilation at night (Benzing and Ott 1981, Cockburn *et al.* 1985, Hew 1987, Hew *et al.* 1984, Hew *et al.* 1991). For most taxa possessing photosynthetic leaves and/or stems carbon assimilation in the roots is generally less than losses due to respiration, resulting in an apparently negative carbon balance for these roots (Dycus and Knudson 1957, Erickson 1957, Hew *et al.* 1984, Kwok-ki *et al.* 1983). In shootless species, where photosynthetic roots represent the sole

means of carbon acquisition for the majority of the plant's life[*] nocturnal carbon assimilation is significantly large enough to result in a net positive carbon balance (Benzing and Ott 1981, Cockburn *et al.* 1985). The magnitude of carbon assimilation in shootless species is generally lower than that of the CAM leaves from other orchids (Benzing and Ott 1981)

Use of radio labeled $^{14}CO_2$ has demonstrated that photosynthetic roots are able to assimilate carbon from the surrounding atmosphere both day and night (Benzing and Ott 1981, Goh *et al.* 1983, Hew *et al.* 1984), suggesting a possible C_3-CAM mode of carbon uptake. The magnitude of diel fluctuations in titratable acidity seem to indicate that for many species potential carbon loss through respiration is mediated by some degree of CAM-cycling (Hew *et al.* 1984). CAM-cycling is further supported by the identification of malate as the product of nocturnal assimilation in species which showed net efflux of CO_2 in the dark (Cockburn *et al.* 1985, Hew *et al.* 1984). This may be evidence of a highly plastic application of the CAM machinery in the photosynthetic roots of orchids.

The presence of a CAM based mode of carbon acquisition and the absence of stomata exhibited by these roots raises some interesting questions. High internal partial pressures of CO_2 are associated with the decarboxylation of malate during the light by CAM plants (Cockburn 1985, Lüttge 1987), yet for several of the photosynthetic roots examined to date net assimilation rates are near zero throughout much of the light (Corckburn *et al.* 1985, Benzing and Ott 1981). How then do these orchids prevent the loss of CO_2 during the light period? In addition many of these orchids live high in forest canopy where water availability is sporadic and evaporative demand may be quite high

[*] Like many orchids these species often posses photosynthetic inflorescences and fruit, capable of off setting much of their carbon costs (Zotz *et al.* 2003). In addition young plants of some shootless orchids may possess small green leaves which are lost with age (Cralsward 2004).

(Benzing 1990), yet they often possess a multitude of aerial roots exposed to the surrounding atmosphere. How then do these plants mediate water loss under these seemingly water demanding conditions? A question even more perplexing for shootless taxa which lack the stems modified for water storage present among other epiphytic orchids.

It has been proposed that specialized aeration complexes present in many orchid roots may provide a regulatory mechanism over gas exchange (Benzing *et al.* 1983), though experimental evidence supporting or refuting this are scanty (Benzing *et al.* 1983, Cockburn *et al.* 1985). These structures which span the exodermis-velamen complex contain two differential thickened cortical cells which may act in much the same manner as stomatal guard cells (Benzing *et al.* 1983). It is the purpose of this report to provide evidence either for or against the presence of a regulatory mechanism based on detailed examination of diel gas exchange patterns and through estimations of internal conductance based on a quantitative model describing water vapor flux.

Materials and Methods

Plant Material

Mature greenhouse grown specimens of *Chiloschista lunifera* (Reichb.f.) J. J. Sm. were obtained from Tropiflora Inc. (Sarasota, Florida). Plants were grown on a course plastic mesh and maintained in the Botany Greenhouse (Department of Botany, University of Florida, Gainesville, Florida) for at least four months prior to use in gas exchange studies. Plants were non-reproductive at the time of measurement, ensuring the roots were the sole means of carbon assimilation and water loss.

Whole Plant Gas-exchange

Diel gas exchange measurements were performed in the lab. Measurements were made on a whole plant basis using open-system analysis with a LI-6400 Portable Photosynthesis System (LI-COR, Inc. Lincoln, Nebraska) fitted with a custom cuvette (20 cm L x 9 cm W x 4cm H), constructed of Polycast acrylic sheet (SPARTECH Corporation, Stamford, Connecticut). Measurements were made over a 24 hour period, with 12 hours of light and 12 hours of darkness, at a constant vapor pressure deficit (VPD) supplied by a LI-610 Portable Dew Point Generator (LI-COR, Inc. Lincoln, Nebraska). Light was supplied by a 50 W halogen lamp mounted 35 cm above the chamber in a darkened room. Flow through the system was held constant at 200 $\mu mol \cdot s^{-1}$, and chamber temperature was held constant by maintaining the block temperature at 27°C. To account for absorption and desorption of water by the acrylic body of the cuvette, a one hour equilibrium period was allowed prior to measurement. Measurements were made at a constant VPD of 2.50, 2.14, 1.78, 1.43 and 1.07 kPa for each 24 hour period, starting at the highest VPD and decreasing stepwise over a period of five days.

All fluxes were calculated on a surface area basis, determined by assuming regular geometry and treating roots as cylinders of constant diameter. Root diameter was determined on a per plant basis as the average diameter of ten roots.

Net assimilation, transpiration and total root conductance were calculated using the standard equations used by the LI-6400 Portable Photosynthesis System (LI-COR 2005).

Water Vapor Flux Across Orchid Velamen

Water vapor flux across excised pieces of velamen was measured using a LI-6400 Portable Photosynthesis System (LI-COR, Inc. Lincoln, Nebraska). Velamen was mounted in a custom cuvette (Fig. 2-1) functionally similar to the porometer chamber described by Meidner (1986), and attached to the LI-6400 sensor head (LI-COR, Inc. Lincoln, Nebraska). The cuvette was designed to replace the lower section of the standard 2X3 Leaf Chamber (LI-COR, Inc. Lincoln, Nebraska). Polycast acrylic sheet (SPARTECH Corporation, Stamford, Connecticut) was used to construct the body of the cuvette. The mounting plate used to hold the velamen in position was constructed from a 0.6 mm thick brass plate, with a 3 mm square orifice machined at the center of the chamber. A water reservoir machined into the lower half of the cuvette provided a water vapor saturated air space on one side of the velamen via a filter paper wick positioned just below the velamen.

Velamen was carefully peeled from healthy living roots at positions well back from the actively growing root tip, using a sharp razor blade. Any remaining cortical material was removed and the velamen sections were blotted dry prior to mounting in the cuvette. Excised pieces of velamen were positioned underneath the mounting plate at its orifice so that the surface formally facing the root cortex faced the water reservoir. The velamen was sealed to the lower half of the cuvette via two closed cell foam rubber gaskets. A type E Chromel-Constantan thermocouple (Omega Engineering, Inc. Stamford, Connecticut) connected to the LI-6400 sensor head was inserted between these gaskets and positioned in the air space under the velamen.

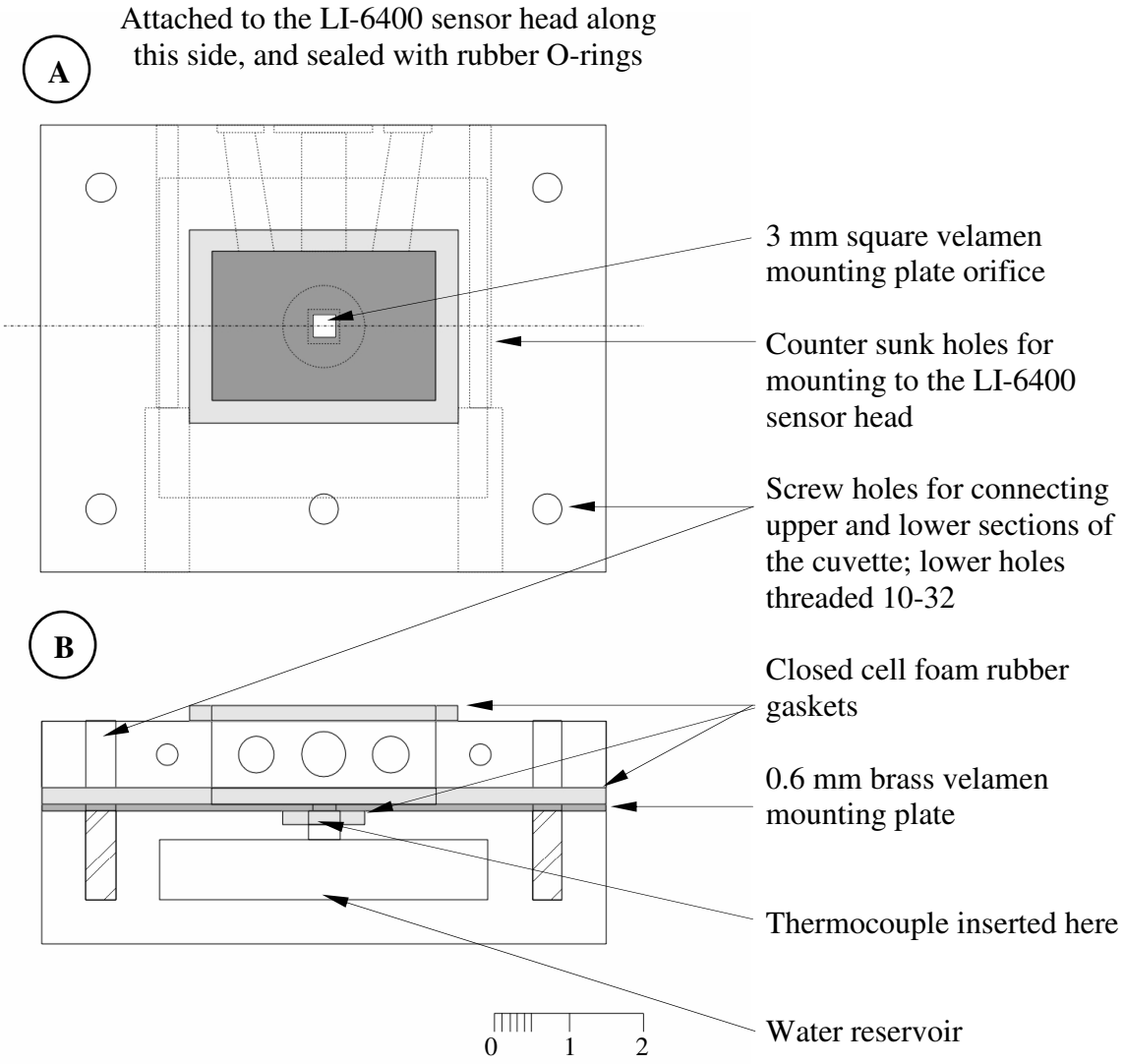

Figure 2-1. Schematic diagram of the velamen cuvette. A. View of chamber from above. B. Cut-away view of chamber along axis drawn through A (heavy dashed line), as viewed from the side. Hidden lines represented as dashed lines. Scale is given in centimeters.

A constant vapor pressure deficit (VPD) was supplied to the cuvette by a LI-610 Portable Dew Point Generator (LI-COR, Inc. Lincoln, Nebraska). Measurements were made on the same piece of velamen at a constant VPD of 2.50, 2.14, 1.78, 1.43 and 1.07 kPa, with a one hour equilibrium period prior to beginning measurement and between

each decrease in VPD. Measurements were logged every 20 minutes for a total of five measurements at each VPD, and the entire experimental procedure was repeated three times. Flow through the system was held constant at 200 μmol·s^{-1}, and chamber temperature was held constant by maintaining the LI-6400's block temperature at 27°C.

It was assumed that the boundary layer conductance for the velamen cuvette, g_{bvc}, represented the combined effects of air passing over the plane of the mounting plate and the unstirred layer of air atop the velamen in the mounting plate orifice (Eq. 2-1) (Nobel 1983).

$$g_{bvc} = \frac{D_w}{\delta_{total}} * \frac{P}{r\,(T_a)} \qquad 2\text{-}1$$

Where D_w is the diffusivity coefficient of water vapor in air (2.4 x10-4 m^2·s^{-1}), and r is the universal gas constant (8.3145 x10^{-3} m^3·kPa·mol^{-1}·K^{-1}). The total boundary layer thickness for the velamen cuvette, $\delta_{total,}$ is calculated from equation 2-2 and expressed in meters (Nobel 1983).

$$\delta_{total} = 4.0\ \sqrt{(L/V)} + D \qquad 2\text{-}2$$

Where L is the characteristic dimension in the direction of air flow and D is the depth of the mounting plate orifice, both expressed in meters. V is the wind speed inside the chamber, expressed in m·s^{-1}. Since velamen mounted in the cuvette is positioned below the bottom plane of the chamber and parallel to the direction of air flow, the entire bottom surface of the chamber contributes to the thickness of the boundary layer, not just the area of exposed velamen at the orifice. So L is equal to 0.02 m, as this is the length of the chamber in the direction of air flow. In addition a contribution 0.0006 m (D) is made

to the boundary layer thickness by the layer of unstirred air positioned atop the velamen in the orifice but below the surface of the mounting plate.

From VP_{vel} (calculated from Eq. 2-4) an estimation of the vapor phase water potential of the velamen, Ψ_{vel}, was made based on the natural log of the ratio of VP_{vel} to the saturation vapor pressure of the surrounding air, SVP_{air}. Following equation 2-3 Ψ_{vel} was calculated and the resulting figure retuned in MPa.

$$\Psi_{vel} = \frac{R\,T_a}{M} \cdot \ln \frac{VP_{vel}}{SVP_{air}} \qquad \text{Eq. 2-3}$$

Where R is the universal gas constant expressed in $J \cdot mol^{-1} \cdot K^{-1}$, T_a is the air temperature inside the velamen cuvette and M is the molecular weight of water (18.02 $g \cdot mol^{-1}$).

Gas-exchange Model

Measurements of excised velamen indicated a capacity of velamen to absorb water vapor directly from moist air, demonstrating a source/sink role of velamen for water vapor moving from the root cortex to the atmosphere. A model describing changes in internal conductance within the root was developed by partitioning water vapor flux to include movement of water into the velamen from both the atmosphere and the root cortex. By assuming the rate of water vapor adsorption and desorption by the velamen is at steady state for a particular vapor pressure inside the velamen, transpiration (E) as determined from the whole plant gas exchange data can be redefined as in equations 2-4 and 2-5.

$$E = g_{bl} \cdot (VP_{vel} - VP_{air}) \qquad 2\text{-}4$$

$$= g_{int} \cdot (VP_{int} - VP_{vel}) \qquad 2\text{-}5$$

Where g_{bl} and g_{int} are the boundary layer and internal conductances respectively, VP_{vel} is the vapor pressure inside the velamen, VP_{int} is the vapor pressure inside the root and VP_{air}

is the vapor pressure of the air surrounding the root. The boundary layer conductance was calculated by assuming regular geometry and treating roots as cylinders oriented perpendicular to the direction of air flow (Nobel 1983). The internal vapor pressure was assumed to be at saturation and was calculated based on measurement of root internal temperature by a type E Chromel-Constantan thermocouple (Omega Engineering, Inc. Stamford, Connecticut) connected to the LI-6400 sensor head and inserted into the root cortex. The vapor pressure of the air surrounding the root was calculated based on the concentration of water vapor in the sample cell of LI-6400 and the air temperature inside the cuvette. By rearranging equations 2-4 and 2-5 to solve for the two remaining unknowns, g_{int} and VP_{vel}, the model used to estimate g_{int} results (Eq. 2-6). This model was used to estimate changes in internal conductance exhibited by *C. lunifera* throughout the course of each diel gas exchange measurement.

$$g_{int} = \frac{1}{\frac{-1}{g_{bl}} + \frac{(VP_{int} - Vp_{air})}{E}} \qquad 2\text{-}6$$

Results

Diel gas exchange patterns for both CO_2 and H_2O were indicative of CAM under all vapor pressure deficits, though the magnitude of these fluxes was quite small under the highest VPD (Fig. 2-2). Net carbon assimilation increased in a stepwise manner as VPD decreased, showing the greatest net carbon gain under the lowest VPD (Fig. 2-3A).

Patterns of water vapor flux were most interesting. Under the highest VPD regime plants exhibited the greatest net water loss, showing what appeared to be a large amount of diurnal variation in transpiration (Fig. 2-2B). These apparent spikes in transpiration at 10:00, 14:00 and 16:00 corresponded to a rapid decrease in transpiration rate following

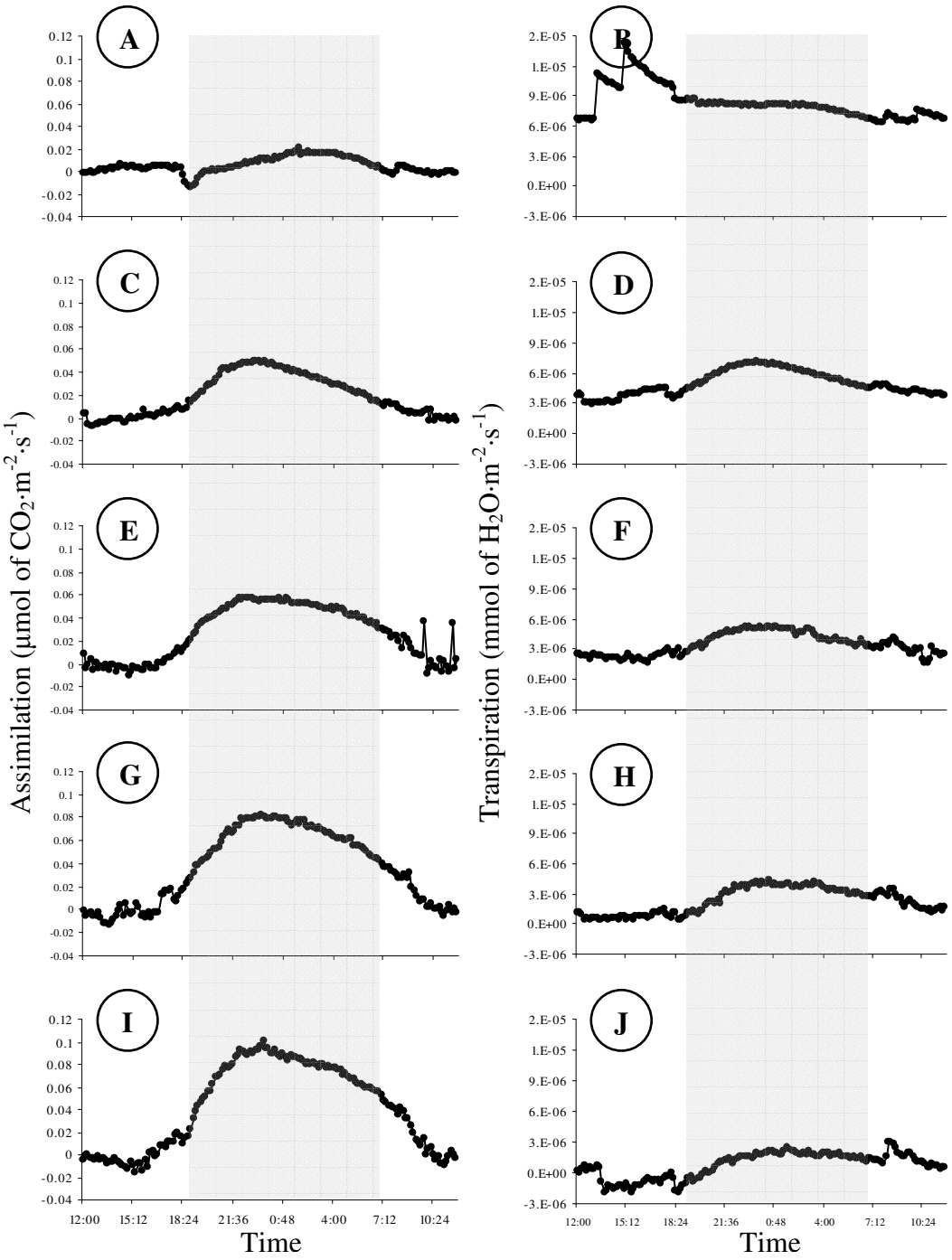

Figure 2-2: Carbon assimilation and transpiration at constant VPD. A. Assimilation at 2.50 kPa. B. Transpiration at 2.50 kPa. C. Assimilation at 2.14 kPa. D. Transpiration at 2.14 kPa. E. Assimilation at 1.78 kPa. F. Transpiration at 1.78 kPa. G. Assimilation at 1.43 kPa. H. Transpiration at 1.43 kPa. I. Assimilation at 1.07 kPa. J. Transpiration at 1.07 kPa. Light period shown in white (~200 µmol·m^{-2}·s^{-1}), dark period shown in gray.

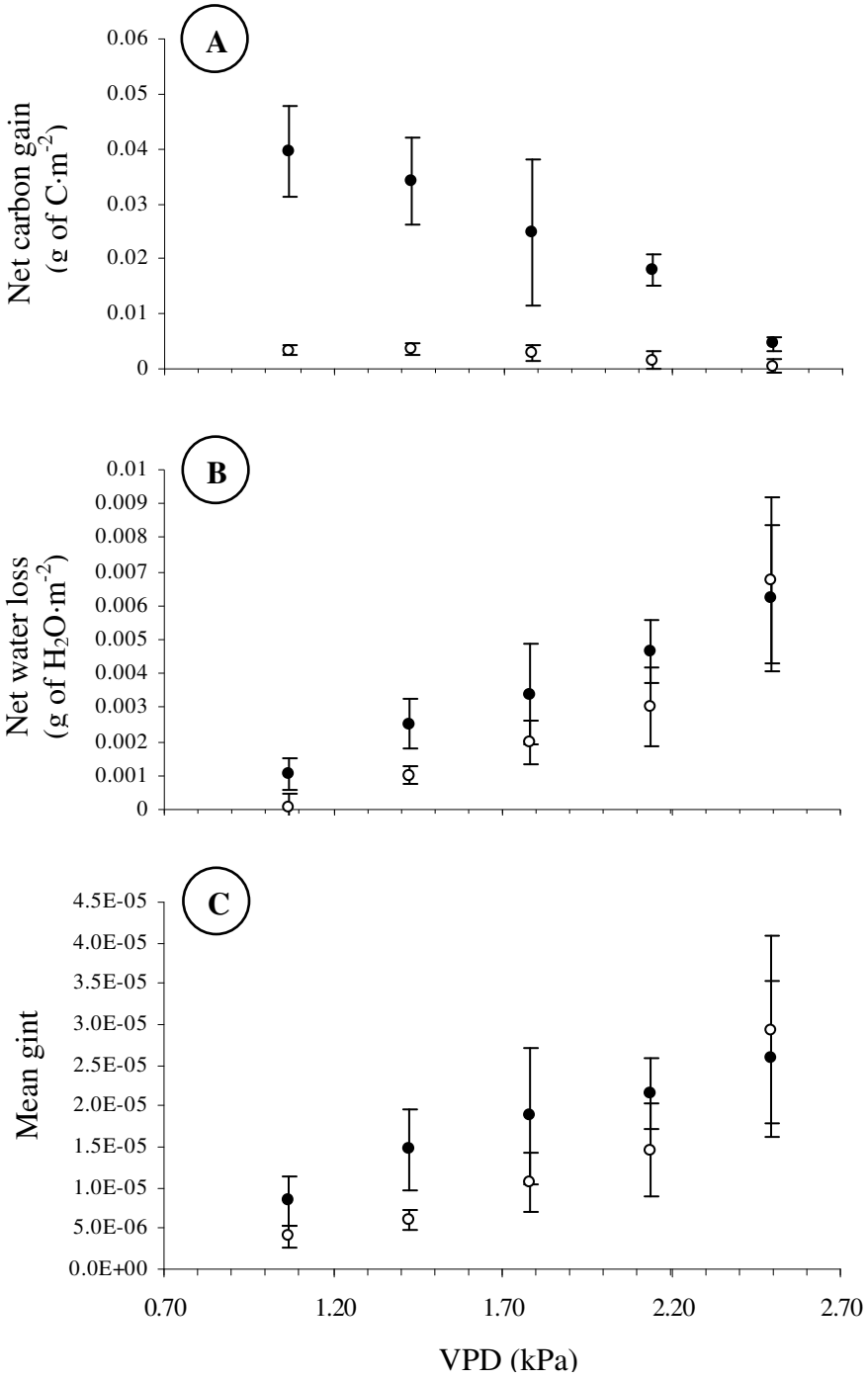

Figure 2-3: Net carbon gain, water loss and internal conductance for all vapor pressure deficits. Net carbon gain (A) and net water loss averaged for all plants (B) integrated for day (open circles) and night (closed circles). C. Internal conductance averaged for day (open circles) and night (closed circles). All values are shown plus or minus standard error.

the start of each new diel gas exchange measurement (Fig. 2-4). Net water loss decreased as VPD decreased, with afternoon transpiration rates gradually approaching zero with each drop in VPD (Fig. 2-3B). Measurements of excised velamen indicated water uptake by this tissue layer at VPDs less than 2 kPa (Fig. 2-5), and this likely played a role in the gradual decrease in transpiration rates. Under the lowest VPD plants exhibited negative transpiration rates for a period of almost 10 hours. This period of water gain by the plants was almost enough to completely counter transpirational losses during the night leading to an almost neutral water balance.

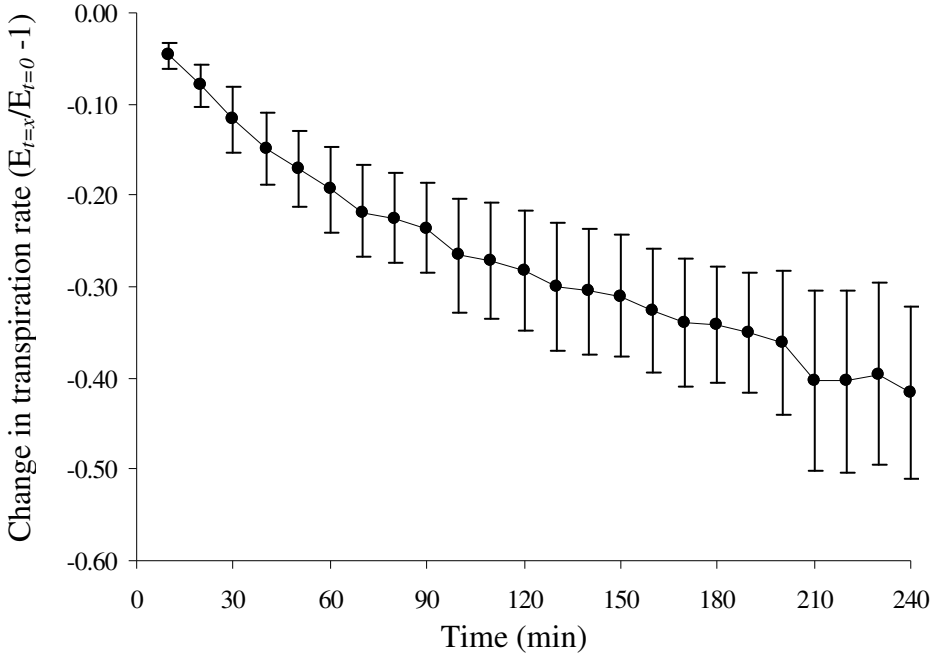

Figure 2-4: Transpiration rate response during the first four hours of measurement at 2.50 kPa VPD. Though the rate of decrease was slightly different between trials, transpiration showed an initial rapid drop off following the start of each set of measurements. Values are shown plus or minus standard error.

Estimations of total root conductance and internal conductance were indicative of CAM under all but the highest VPD (Fig. 2-6) and similar to transpiration, conductance was lower during the day than at night (Fig. 2-3C). The vapor phase water potentials for velamen sheathing the intact root were similar to those determined for isolated velamen mounted in the velamen cuvette under all VPDs (Table 2-1).

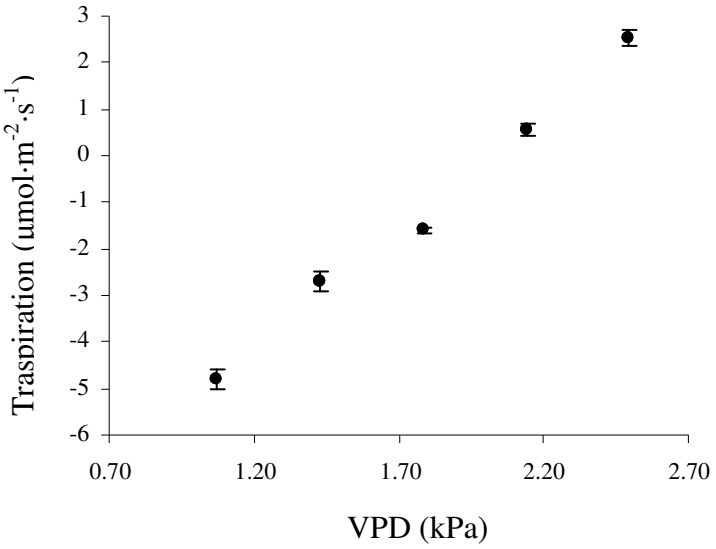

Figure 2-5: Water vapor flux across isolated velamen tissue. The units of transpiration have been converted to $\mu mol \cdot m^{-2} \cdot s^{-1}$ for convenience as flux rates are small. Error bars represent standard error.

Table 2-1: Velamen vapor phase water potential. Ψ_{vel} (MPa) was estimated from measurements of isolated velamen mounted in the velamen cuvette and for intact velamen based on whole plant gas exchange data.

VPD	Velamen cuvette		Whole plant	
	Mean	Std. Error	Mean	Std. Error
2.50	-163.52	± 1.28	-176.66	± 15.37
2.12	-139.88	± 0.95	-136.19	± 1.74
1.78	-107.82	± 0.75	-105.46	± 4.34
1.43	-91.73	± 0.88	-92.19	± 5.52
1.07	-68.52	± 2.27	-66.12	± 3.28

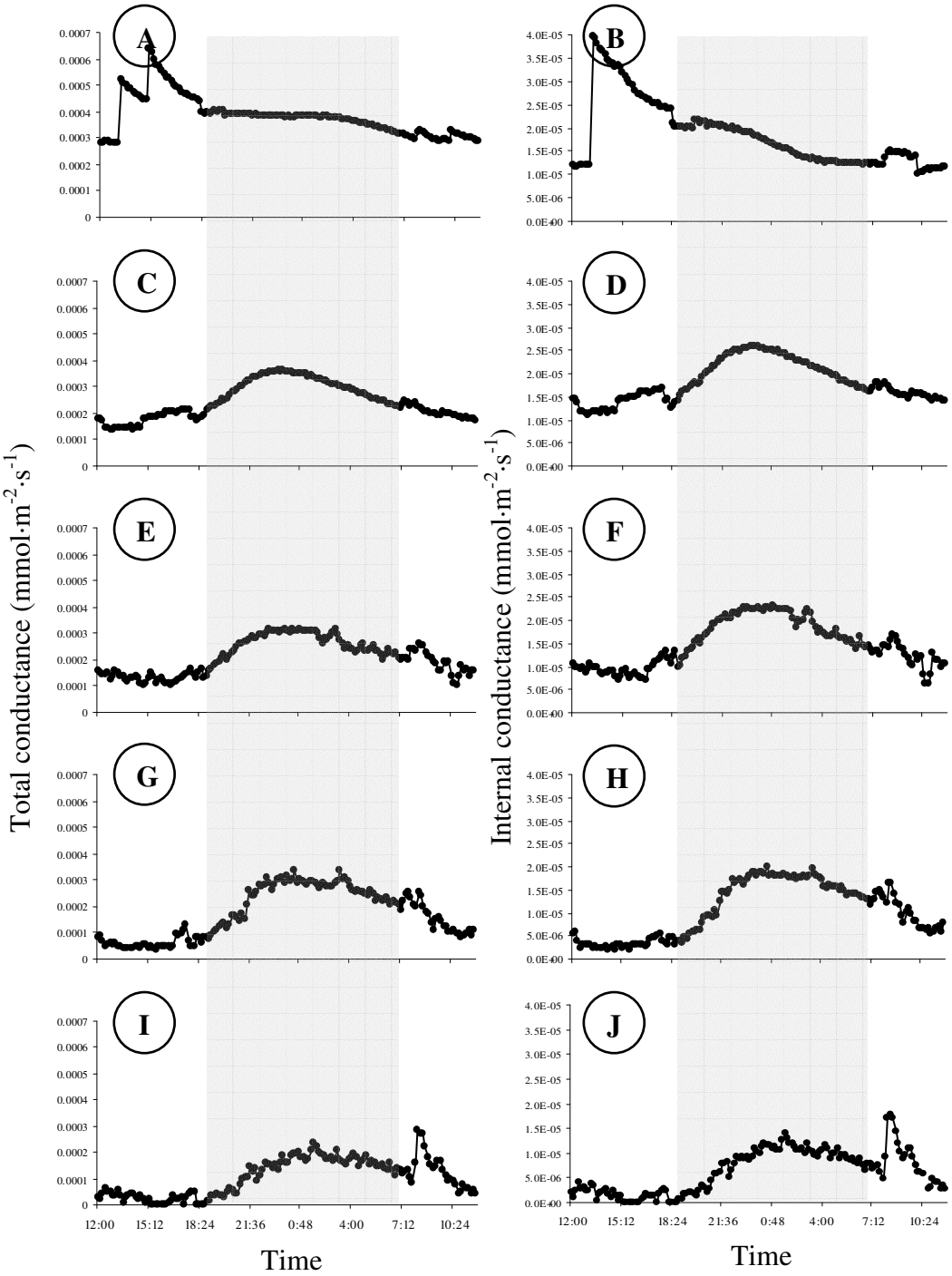

Figure 2-6: Total plant conductance and internal conductance of water vapor at constant VPD. A. Total conductance at 2.50 kPa. B. Internal conductance at 2.50 kPa. C. Total conductance at 2.14 kPa. D. Internal conductance at 2.14 kPa. E. Total conductance at 1.78 kPa. F. Internal conductance at 1.78 kPa. G. Total conductance at 1.43 kPa. H. Internal conductance at 1.43 kPa. I. Total conductance at 1.07 kPa. J. Internal conductance at 1.07 kPa. Light period shown in white (~200 μmol·m^{-2}·s^{-1}), dark period shown in gray.

Discussion

The rates of carbon assimilation in photosynthetic orchid roots have been demonstrated to be quite low, similar to those rates observed in the present work (Benzing and Ott 1981, Cockburn *et al.* 1985, Dycus and Knudson 1957, Erickson 1957, Hew *et al.* 1984, Kwok-ki *et al.* 1983). Consistent with the results reported for other shootless species, *Chiloschista lunifera* exhibited a strong CAM signal at night and showed little or no carbon efflux during the day (Cockburn *et al.* 1985). While *C. lunifera* showed diel gas exchange patterns for CO_2 similar to those reported for other shootless orchids, these results and those published by other authors (Benzing and Ott 1981, Cockburn *et al.* 1985) by themselves shed little light on a possible regulatory mechanism over gas exchange.

Diel changes in transpiration rates tracked that of assimilation, showing maximum transpiration rates at night when transpirational demands were presumably the lowest. This change in transpiration could not be attributed to any change in the forces driving water vapor flux from the roots of *C. lunifera* as measurements were made under constant temperature and VPD, nor could they be explained by evaporation of water absorbed by the velamen. Estimations of internal conductance tracked transpiration and provide evidence that some mechanism inside the plant must exist to regulate gas exchange. Without such a mechanism it seems highly unlikely that any change in diel transpiration patterns would be observed in *C. lunifera* under these measurement conditions, or if transpiration rates were to show diel variation, maximum rates would be expect during the day do to the energy contributed to the system by light striking the plant.

In addition to diel variation in transpiration, *C. lunifera* showed an immediate response to a sudden large scale change in VPD. Plants were placed in the cuvette for measurement directly from the greenhouse, moving from the relatively moist cool conditions of the greenhouse to the much drier conditions used for the first set of diel gas exchange measurements (VPD of 2.50 kPa). Plants exhibited a fairly rapid decline in transpiration rate, with transpiration rates on average reduced by 40% within 3.5 hours after the start of measurement. As with the diel variation observed in transpiration rates, this decline in transpiration rate can not be explained by changes in the forces driving water vapor flux from the roots of *C. lunifera*, nor by water adsorption or desorption by the velamen. Similar responses have been observed for other plant organs in response to decreased humidity during gas exchange measurements and have been ascribed to changes in stomatal conductance (Schulze *et al.* 1987). It seems likely that this is evidence that some mechanism exists within the roots of *C. lunifera* that allows the plant to regulate gas exchange.

The overall drop in transpiration with each decrease in VPD, and the eventual ten hours of apparent water vapor uptake by the plants is likely due to the ability of velamen to adsorb water vapor from the surrounding air (Giles and Agnihotri 1968). The magnitude of water vapor fluxes into the velamen and out of the root are both small, and at the lowest VPD these fluxes were nearly equal when integrated over a 24 hour time period, leading to a near neutral water balance. However, it remains to be seen if the water vapor absorbed by the velamen remains bound there or is able to be pulled into the root cortex and used by the plant.

The anatomy of roots from many shootless taxa has been well defined (Benzing *et al.* 1983, Carlsward 2004). Within these roots specialized aeration complexes exist spanning the exodermis and velamen, which presumably provide a pathway for diffusive gas exchange (Benzing *et al.* 1983, Cockburn 1985). These complexes are comprised of a highly suberised region in the velamen, the pneumathode, which remains void even when other velamen cells are fully saturated (Benzing and Ott 1981, Benzing *et al*. 1983, Dycus & Knudson 1957, Pridgeon 1987), an eroded aeration cell spanning the exodermis, and two differential thickened cortical cells subtending the aeration cell (Benzing and Ott 1981, Benzing *et al.* 1983, Carlsward 2004). It has been proposed that these cortical cells may act analogously to stomatal guard cells to provide a means of regulating gas exchange in orchid roots (Benzing *et al.* 1983). These structures are present in the roots of *C. lunifera* (Carlsward 2004), and represent the most likely means by which *C. lunifera* could regulate gas exchange. Diel transpiration patterns, the lack of CO_2 evolution during the day and the decrease in transpiration rates following the drastic increase in VPD at the start of measurement are all indicative of some regulatory mechanism over gas exchange. The variation in transpiration throughout the day and the observation that transpiration rates tracked those of assimilation suggest a stomata like mechanism under active control of the plant.

The overall higher transpiration rates at the higher VPDs may be the result of desorption of water vapor from the velamen following movement from the moist greenhouse to the much drier cuvette, or they maybe evidence that while a regulatory mechanism exists it does not provide tight control over gas exchange. Estimations of vapor phase water potential of the velamen are very negative at these VPDs, suggesting

the velamen tissue is dry. It therefore seems highly probable that the mechanism used to regulate gas exchange in *C. lunifera* is crude and is not capable of completely closing off the root cortex from the surrounding atmosphere.

It is clear from the data that a regulatory mechanism exists that allows *C. lunifera* to exert control over fluxes of CO_2 and water vapor in and out of its roots. While the mechanism does not appear to be as refined as that seen in other plants (e.g. stomata), it does appear to be under active control of the plant. It is likely that further investigation will reveal that the observed changes in transpiration rates throughout the day correspond to changes in cortical component aperture.

APPENDIX

GLOSSARY OF SYMBOLS

Symbol	Description	Units
D	Mounting plate orifice depth	m
D_w	Diffusivity coefficient of water in air (2.4×10^{-4})	$m^2 \cdot s^{-1}$
E	Transpiration rate	$mmol \cdot m^{-2} \cdot s^{-1}$
g_{bl}	Boundary layer conductance of water vapor	$mmol \cdot m^{-2} \cdot s^{-1}$
g_{bvc}	Boundary layer conductance for velamen mounted in the velamen cuvette	$mmol \cdot m^{-2} \cdot s^{-1}$
L	Characteristic dimension	m
M	Molecular weight of water (18.02)	$g \cdot mol^{-1}$
P	Atmospheric pressure	kPa
r	Universal gas constant (8.3145×10^{-3})	$m^3 \cdot kPa \cdot mol^{-1} \cdot K^{-1}$
R	Universal gas constant (8.3145)	$J \cdot mol^{-1} \cdot K^{-1}$
r_{bl}	Boundary layer resistance to water vapor flux	
r_{exo}	Resistance to water vapor flux due to the exodermis	
r_{pnue}	Pneumathode resistance to water vapor flux	
T_a	Air temperature inside the cuvette	K
T_r	Root temperature	C
V	Wind speed	$m \cdot s^{-1}$
VP_{air}	Water vapor pressure of air	kPa
VP_{int}	Water vapor pressure inside the root	kPa

VP_{vel}	Water vapor pressure of velamen tissue	kPa
δ_{total}	Total boundary layer thickness for velamen mounted in the velamen cuvette	m
Ψ_{vel}	Vapor phase water potential of the velamen	MPa

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BIOGRAPHICAL SKETCH

Jason Robert Hupp was born in Lexington, North Carolina, on October 15, 1980. He graduated from Mooresville Senior High School, in 1998. The following spring he enrolled at Catawba College, in Salisbury, North Carolina, and began working under the guidance of Michael J. Baranski. Jason received his Bachelor of Science degree in 2003, after completing the requirements for both a major in biology and in environmental science, and a minor in chemistry. In the fall of 2003 he relocated to Gainesville, Florida, and began work on a master's degree at the University of Florida under the supervision of Stephen S. Mulkey.