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Importancia de la conductancia del mesófilo sobre la fotosíntesis de plantas alto-andinas en Chile central: evaluando el rol de la temperatura ambiental

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Resumen

Esta tesis doctoral explora el papel de la conductancia mesofílica (g_m) en los procesos fotosintéticos de plantas alpinas en diferentes elevaciones del centro de Chile. La importancia de la g_m radica en su impacto directo sobre la difusión de CO_2 dentro del mesófilo de la hoja, lo que es un determinante crucial de las tasas fotosintéticas. Al centrarse en plantas zonales y azonales, este estudio investiga las adaptaciones fisiológicas y morfológicas asociadas a la asimilación de carbono, que facilitan la supervivencia y productividad bajo las condiciones ambientales únicas de diferentes plantas de gran altitud. También, se examina específicamente cómo la g_m responde a la temperatura de crecimiento y contribuye a las capacidades adaptativas de las plantas alpinas. Se evalúan las diferencias en la respuesta de la g_m entre plantas zonales y azonales y sus estrategias ecológicas únicas de acuerdo con su entorno particular. Las hipótesis de este trabajo son: (1) la fotosíntesis no muestra diferencias altitudinales debido a mecanismos compensatorios, como una mayor densidad estomática (SD) en plantas tanto zonales como azonales. En las plantas zonales, la masa foliar por área (LMA) y la limitación del mesófilo no cambian con la altitud, ya que tanto los hábitats de gran altitud como los de baja altitud presentan condiciones ambientales adversas, como bajas temperaturas y baja humedad del suelo, respectivamente. En contraste, para las plantas azonales, existe un mayor LMA y una mayor limitación del mesófilo a gran altitud. (2) Los cambios de g_m ante distintas condiciones ambientales se deben a cambios anatómicos como el grosor de la pared celular y la disposición de los cloroplastos. (3) La temperatura es el factor que impulsa las tasas fotosintéticas más bajas en las plantas de gran altitud y el mecanismo subyacente a las variaciones en la fotosíntesis neta ($A_{N)}$ asociadas a la temperatura involucra cambios en el LMA y, en consecuencia, en la g_m. Los resultados sugieren que la g_m no solo es el principal factor limitante en la difusión de CO_2 en las plantas alpinas, sino también un rasgo dinámico que varía significativamente con la elevación, el tipo de planta y la temperatura. Además, se observó cómo las variaciones en el grosor de la pared celular y la disposición de los cloroplastos se correlacionan con los cambios en g_m.

A través de este análisis exhaustivo, el estudio no solo contribuye a nuestra comprensión de la fisiología vegetal, sino que también mejora nuestro conocimiento sobre cómo los ecosistemas alpinos pueden funcionar en respuesta a sus condiciones ambientales. En conclusión, esta investigación ofrece valiosos conocimientos sobre los procesos fundamentales de adaptación de las plantas a grandes altitudes, destacando el papel crítico de la conductancia mesofílica. Establece las bases para futuros estudios ecológicos y fisiológicos, con el objetivo de dilucidar las complejas interacciones entre la morfología vegetal, los rasgos fisiológicos y los factores ambientales en las regiones montañosas. Las implicaciones de estos hallazgos son significativas para contribuir a predecir las respuestas de la vegetación alpina a los cambios ambientales y para la conservación y gestión de estos ecosistemas sensibles.

Abstract

This doctoral thesis explores the role of mesophyll conductance (g_m) in the photosynthetic processes of alpine plants at different elevations in central Chile. The importance of g_m lies in its direct impact on CO_2 diffusion within the leaf mesophyll, which is a crucial determinant of the photosynthetic rates of alpine vegetation. By focusing on both zonal and azonal plants, this study investigates the physiological and morphological adaptations that facilitate survival and productivity under the unique environmental conditions of different high-altitude ecosystems. It also specifically examines how g_m responds to growth temperature and contributes to the adaptive capacities of alpine plants. Differences in g_m responses between zonal and azonal plants are highlighted, revealing how these groups exhibit unique ecological strategies according to their specific environments. The hypotheses of this study are: (1) Photosynthesis does not show altitudinal differences due to compensatory mechanisms such as higher stomatal density (SD) in both zonal and azonal plants. In zonal plants, LMA and mesophyll limitation do not change with altitude, as both high- and low-altitude habitats present adverse environmental conditions, such as low temperatures and low soil moisture, respectively. In contrast, azonal plants exhibit higher LMA and greater mesophyll limitation at high altitudes. (2) Changes in g_m under different environmental conditions are due to anatomical modifications such as cell wall thickness and chloroplast arrangement. (3) Temperature is the driving factor behind lower photosynthetic rates in high-altitude plants, and the mechanism underlying temperature-associated variations in A_N involves changes in LMA and, consequently, in g_m. The results suggest that g_m is not only the main limiting factor in CO2 diffusion in alpine plants but also a dynamic trait that varies significantly with elevation, plant type, and temperature. Additionally, this thesis delves into the anatomical traits that support these responses. Variations in cell wall thickness and chloroplast arrangement were found to correlate with changes in g_m. Through this comprehensive analysis, the study not only contributes to our understanding of plant physiology but also enhances our knowledge of how alpine ecosystems function in response to their environmental conditions. In conclusion,

this research provides valuable insights into the fundamental adaptation processes of plants at high altitudes, highlighting the critical role of mesophyll conductance. It lays the foundation for future ecological and physiological studies aimed at elucidating the complex interactions between plant morphology, physiological traits, and environmental factors in mountainous regions. The implications of these findings are significant for predicting the responses of alpine vegetation to environmental changes and for the conservation and management of these sensitive ecosystems.

Introducción general

La fotosíntesis es el proceso mediante el cual, gracias a la energía solar, el carbono inorgánico proveniente del aire se transforma en carbono orgánico. La fotosíntesis es la base de las cadenas tróficas del planeta, lo que la transforma en uno de los procesos más importantes junto con la respiración.

Para que se produzca la fotosíntesis, el CO_2 debe entrar a la hoja a través de los estomas, pasando desde un medio gaseoso a uno acuoso. Entre las barreras que debe atravesar se encuentra la pared celular, la membrana plasmática, el citosol, la membrana externa e interna del cloroplasto, hasta llegar al estroma donde es fijado enzimáticamente por acción de la enzima Ribulosa 1,5 bifosfato carboxilasa oxigenasa (RuBisCO). Así, los determinantes que mayormente controlan la fotosíntesis en las plantas se han clasificado en tres grandes grupos: las limitaciones asociadas a la conductancia estomática (g_s), la conductancia del mesófilo (g_m) y la capacidad bioquímica (Gago et al. 2019).

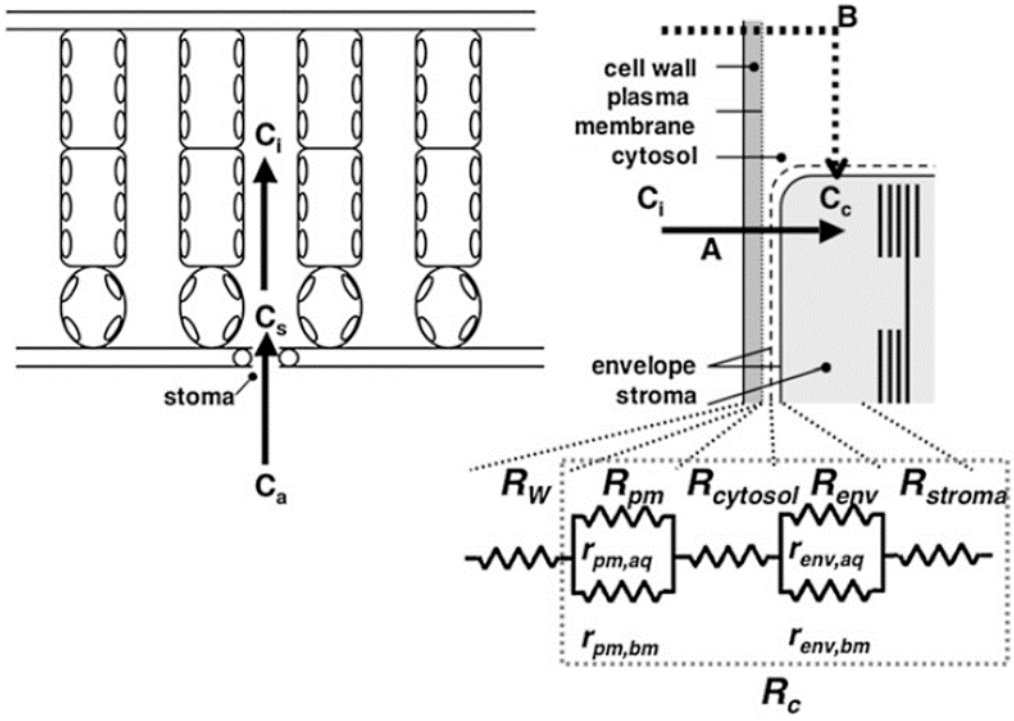

Fig. 1. Esquema de la anatomía de la hoja y difusión del aire fuera de la hoja hasta el estroma del cloroplasto. C denota las distintas concentraciones de CO_2: en el ambiente (Ca), dentro de la cavidad subestomática (Cs), dentro de los espacios intercelulares (Ci) y dentro del estroma (Cc). El panel de la derecha muestra un acercamiento de una célula del mesófilo expuesta a espacios intercelulares y los diferentes componentes de ella de arriba hacia abajo son: Pared celular, membrana plasmática, citosol y envoltura del estroma. El panel de abajo muestra la resistencia del mesófilo (R) por unidad de área del cloroplasto expuesto a la superficie (g_m /sc). El flujo de CO_2 por la ruta B es despreciable comparado con el de la ruta A debido a que en condiciones fisiológicas la difusión de carbono inorgánico en este medio (citosol) es muy lenta. Imagen obtenida desde (Terashima et al. 2011).

Limitaciones por conductancia estomática

El comportamiento estomático es uno de los factores más importantes que controlan la fotosíntesis, pues controla el intercambio de gases como CO_2 y H_2O entre las hojas y el aire. Los estomas están formados por las células oclusivas que se ubican en la epidermis de la hoja y que delimitan entre ellas un poro llamado ostiolo. Estas células junto con las células acompañantes forman el aparato estomático, que normalmente se abre a una cavidad debajo de la epidermis llamada cámara subestomática, cuya función es ser la puerta de entrada y salida de los gases necesarios para el metabolismo fotosintético. A la conductancia del CO_2 a través de los estomas se le denomina conductancia estomática (g_s) (Carpenter, 2005).

Cuando los estomas se abren para permitir la entrada del CO_2, la hoja comienza a liberar agua hacia la atmósfera. Si las raíces no son capaces de suplir esta pérdida de agua, disminuye el contenido relativo de agua de la hoja (RWC) lo cual resulta perjudicial para la planta. Otro factor importante para la conductancia estomática es el déficit de presión de vapor de agua (VPD en inglés). Este concepto se relaciona con la cantidad de agua necesaria para saturar la atmósfera, en otras palabras, la cantidad de agua que puede retener la atmósfera, este valor depende

del contenido de agua en el ambiente y la temperatura. El VPD es importante para la transpiración de las plantas, ya que cuando aumenta la diferencia entre la hoja y el aire, las plantas liberarán más vapor de agua por los estomas. Al haber más transpiración habrá mayor absorción de nutrientes y fotosíntesis (Cramer et al. 2007). Sin embargo, si la pérdida de agua es excesiva la planta tendrá una serie de respuestas fisiológicas que llevarán al cierre de sus estomas, impidiendo también la entrada de CO_2.

Una consecuencia del control de la apertura estomática es la mayor eficiencia en el uso del agua. Se ha observado que ante una misma disminución de la presión parcial de CO_2 en las cavidades subestomáticas, la fotosíntesis disminuye en menor proporción que la transpiración, esto resulta en un mayor número de moléculas de carbono fijadas por unidad de agua perdida (Lambers et al. 2019). A pesar de la inicial mayor eficiencia en el uso del agua con menor conductancia a través de los estomas, la disminución de la presión parcial de CO_2 en las cavidades subestomáticas produce un menor suministro de CO_2 a los cloroplastos y eventualmente una limitación a la fotosíntesis ante el déficit hídrico (Flexas et al. 2004).

Limitaciones por conductancia del mesófilo

La conductancia del mesófilo se define como la eficiencia de difusión de CO_2, desde la cámara subestomática y los espacios intercelulares hasta los sitios activos de la RuBisCO. De este modo, la conductancia del mesófilo incluye la difusión a través de las paredes celulares, membranas plasmáticas, citosol, envoltura externa e interna de los cloroplastos y estroma (Flexas et al. 2018). La ley física que rige la resistencia y la conductancia en estos casos es la ley de Fick, la cual afirma que la conductancia depende de: 1) la difusividad de la sustancia (en este caso CO_2), 2) la temperatura (por su efecto sobre la presión parcial), 3) el medio en que ocurre la difusión, y 4) la distancia de difusión. Cuando el CO_2 pasa desde los espacios intercelulares aéreos a la superficie externa de las células del mesófilo existe un cambio de fase desde una gaseosa a una líquida, la difusión de este gas en fase

gaseosa es 104 veces mayor a la que ocurre en fase líquida por lo que esta última limita en mayor medida la conductancia del CO_2 a través del mesófilo (g_m) (Evans et al. 2009).

Se ha demostrado que la g_m está determinada tanto por componentes anatómicos como no anatómicos. Estudios recientes han encontrado discrepancias entre la g_m calculada a partir de anatomía y la estimada por métodos de intercambio de gases. Estas diferencias sugieren la existencia de mecanismos facilitadores de la difusión de CO_2 como podrían ser la actividad de acuaporinas y anhidrasa carbónica (Fabre et al. 2007; Terashima & Ono 2002). La anhidrasa carbónica es una enzima que cataliza las interconversiones de CO_2 a HCO_3. Esto es importante ya que gran parte del CO_2 se transforma en HCO_3 cada vez que entra en un medio acuoso, y, por otro lado, cuando pasa a través de una membrana lipídica lo hace transportado principalmente como CO_2. Debido a esto, la anhidrasa carbónica facilita la llegada del CO_2 hasta la RuBisCOy se ha demostrado que en algunos casos su actividad esta correlacionada con la g_m (Momayyezi & Guy 2017).

En la fase líquida, la pared celular parece ser la barrera más limitante para la conductancia del CO_2. Terashima y colaboradores (2011) encontraron una relación inversa entre el grosor de la pared celular y la conductancia del mesófilo. Después de atravesar la pared celular, el CO_2 debe atravesar una fase lipídica, la membrana plasmática, difusión que estaría facilitada por poros de membrana capaces de transportar CO_2, llamados acuaporinas. Estas proteínas se han relacionado a variaciones rápidas de la conductancia del mesófilo en respuesta a cambios ambientales. Los cambios en la g_m relacionados a la expresión de acuaporinas han sido observados a través de técnicas de transformación genética en plantas de tabaco donde mutantes con una menor expresión de acuaporinas presentaban una menor conductancia del mesófilo y un menor crecimiento (Flexas et al. 2006).

Una vez atraviesa la membrana plasmática, el CO_2 debe difundir a través del citosol hasta los cloroplastos, esta distancia de difusión puede ser mínima si los cloroplastos se encuentran adyacentes a la superficie del mesófilo expuesta a espacios intercelulares aéreos. Diferentes estudios han reportado una correlación

positiva entre la g_m y la superficie de mesófilo expuesto a espacios intercelulares aéreos ocupada por cloroplastos (Sc/Sm) (Terashima et al. 2011; Tholen et al. 2008). Por ejemplo, en *Populus tremula*, se observó como la capacidad fotosintética se relaciona con la conductancia del mesófilo a través de características anatómicas como: la superficie de cloroplastos expuesta a espacios intercelulares por área (Sc), el grosor de la pared celular, los espacios internos aéreos y el tamaño de los cloroplastos (Tosens et al. 2011).

Limitaciones bioquímicas

Una vez llega el CO_2 a los cloroplastos, la incorporación a la ribulosa-1,5-bisfosfato (RuBP) da inicio al ciclo de Calvin, lo que permite la formación de moléculas orgánicas necesarias para las plantas. La regulación de este proceso depende de distintos ciclos bioquímicos y puede estar limitada de diferentes maneras. En la gran mayoría de las plantas vasculares, la fijación del carbono en compuestos orgánicos ocurre cuando el CO_2 se une a RuBP, en una reacción catalizada por RuBisCO, formando 2 moléculas de ácido 3-fosfoglicérico (PGA), este proceso utiliza NADPH y ATP proveniente de las reacciones fotoquímicas de los tilacoides del cloroplasto (Lambers et al 2019). Acorde a esto, existen 3 variables de la cinética bioquímica de las plantas que determinan sus tasas de fotosíntesis, ellas son: (1) La velocidad máxima de carboxilación (Vcmax), que denota la actividad de la RuBisCO: el valor de esta variable será bajo si existe una menor Kcat (número de recambio) y/o concentración de sitios activos de la enzima, lo cual podría disminuir la fotosíntesis; (2) La tasa máxima de transporte de electrones (Jmax), relacionado a la capacidad de transporte de electrones necesarios para la regeneración de la RuBP: una baja disponibilidad lumínica o capacidad de transporte de electrones podría ser otra limitante bioquímica para la fotosíntesis; y finalmente (3) la velocidad de utilización de las triosas fosfato en la síntesis de sacarosa y almidón (VTPU), valor asociado a la capacidad de utilización de PGA: si existe una baja demanda de productos de la fotosíntesis este proceso tenderá a detenerse por exceso de producto y un

insuficiente intercambio de fósforo inorgánico (Long & Bernacchi 2003; Sage & Kubien 2007).

Un último factor que podría limitar la capacidad bioquímica de las plantas es la especificidad de la RuBisCO (Sc/o). Esta enzima no es catalíticamente eficiente ya que el O_2 compite con el CO_2 por los sitios activos de esta enzima, que tiene actividad carboxilasa y oxigenasa. La similitud molecular entre los dos gases (CO_2 y O_2), fuerza a la RuBisCO a un compromiso entre la rapidez y la especificidad por el sustrato (Tcherkez et al. 2006). Por lo que a una baja especificidad por CO_2 o concentración de éste podría disminuir la actividad carboxilasa de la RuBisCO.

De acuerdo con toda la evidencia anterior, la fotosíntesis puede ser limitada principalmente en tres procesos fundamentales: la conductancia a través de los estomas, la conductancia a través del mesófilo de la hoja y finalmente los procesos bioquímicos involucrados en la fijación de CO_2 en moléculas orgánicas. Estos tres procesos tienen distintos determinantes que en conjunto nos darán información sobre: por qué las plantas pueden asimilar más o menos carbono para realizar sus procesos vitales, y también, sobre cómo la proporción de estas limitaciones cambia dependiendo de las condiciones ambientales, en favor de mantener mayores tasas fotosintéticas.

Efectos del ambiente sobre la fotosíntesis

Para compensar los efectos de cambios en las condiciones ambientales o la presencia de estresores, las plantas modifican su funcionamiento interno con lo cual logran mantenerse con vida y desarrollarse. Así, las plantas vasculares son capaces de tener balances positivos de carbono y crecer en condiciones adversas, como las presentes en un desierto cálido y seco o una zona cercana a los polos con bajas temperaturas. En cada lugar del planeta en que crecen plantas, existen condiciones ambientales particulares al igual que adaptaciones específicas para estas condiciones, lo que determina finalmente que las plantas estén limitadas en sus procesos fotosintéticos de distintas formas.

Bajas temperaturas

Uno de los mayores determinantes de la fotosíntesis en plantas es la temperatura. Se ha reportado que la asimilación de carbono puede realizarse entre 0 y 30°C en plantas adaptadas al frío, como las que crecen a altas elevaciones y latitudes (Larcher & Poorter 2004). Para lograr esto las plantas experimentan un proceso de aclimatación a las bajas temperaturas. Este proceso se ha asociado bioquímicamente a una mayor sensibilidad de la fotosíntesis a los cambios de CO_2 y O_2 lo cual denotaría una baja limitación bioquímica por regeneración de fosforo inorgánico (VTPU) (Sage & Kubien 2007). Esto podría ocurrir debido a que las plantas que crecen en ambientes fríos dedican a una gran parte de sus productos fotosintéticos a la formación de azucares de reserva, útiles durante el invierno cuando disminuyen las tasas de fotosíntesis. Acorde a esto, se ha mostrado cómo las plantas aclimatadas al frío presentan mayores concentraciones de sacarosa en el floema (Strand et al. 1999). Otra limitación bioquímica para las plantas de ambientes fríos es la disminución de la velocidad con que la RuBisCO transforma las moléculas de sustrato (Kcat). Para compensar esta menor Kcat, las plantas usualmente aumentan sus cantidades de enzimas o sintetizan isoformas que se desempeñan mejor a bajas temperaturas (Huner & Macdowall 1979; Yamori et al. 2006) haciendo que la Vcmax no disminuya o incluso sea mayor en plantas aclimatadas al frío.

En cuanto al transporte de electrones cloroplástico, en plantas que crecen a bajas temperaturas, no existe un patrón claro. En varias especies se habría observado un ajuste del óptimo térmico tanto para la fotosíntesis como el transporte de electrones (Sage & Kubien 2007). Así mismo, Yamori y colaboradores (2008) muestran en hojas crecidas a 30°C versus 15°C como el transporte de electrones es menos limitante a menores temperaturas. Debido a esto la capacidad bioquímica de las hojas aclimatadas al frío parece responder de buena forma ante la disminución de las temperaturas y podría verse afectada solo ante condiciones

extremas, lo cual podría significar que ante condiciones de frío la limitación bioquímica no sería predominante.

Se ha propuesto que ante la disminución de las temperaturas los factores no estomáticos son los mayores responsables de la disminución de la fotosíntesis (Flexas et al. 2014). Esto podría deberse a que físicamente, las bajas temperaturas disminuyen el delta de VPD entre la hoja y el ambiente por lo que disminuye la demanda evaporativa de las plantas (Wang et al. 2017), así como también existe una menor evaporación de agua en el suelo permitiendo una mayor disponibilidad para las raíces.

Dado que las bajas temperaturas parecen no disminuir drásticamente las limitaciones estomáticas y bioquímicas, la disminución de las tasas de fotosíntesis en plantas que crecen en frío podrían deberse a la disminución de la conductancia del CO_2 a través del mesófilo. Experimentos de laboratorio como los realizados por Yamori y colaboradores (2006) han mostrado evidencia de esto. Utilizando plantas de *Spinacia oleracea* crecidas a 15 y 30 °C, encontraron que las hojas crecidas a menor temperatura presentaban una menor presión parcial interna de CO_2 y una mayor limitación por conductancia del mesófilo. En ambientes naturales, Sáez y colaboradores (Sáez et al. 2017; 2018) mostraron la gran importancia de la conductancia del mesófilo como limitante a la fotosíntesis en plantas vasculares de la antártica que crecen a bajísimas temperaturas. Interesantemente, esta baja conductancia fue compensada en parte por una alta eficiencia bioquímica. Onoda y colaboradores (2017) explican que podría existir un compromiso entre la capacidad de hacer fotosíntesis y la resistencia a climas extremos como los que presentan bajas temperaturas. En estas situaciones las plantas invertirían más en pared celular lo cual les conferiría mayor resistencia a su ambiente a cambio de una menor difusión de CO_2 y por ende menor fotosíntesis. Por otro lado, esta tendencia podría no ser similar en todas las plantas. Caemmerer y Evans (2015) afirman que la respuesta de la g_m a la temperatura es especie específica. En sus experimentos observaron que, ante la disminución de la temperatura, la g_m de plantas del género *Nicotiana* disminuye a menos de la mitad, mientras que en otras plantas del género

Lophostemon existen solo cambios marginales. Así mismo, experimentos en plántulas de *Eucalyptus regnans* no encontraron efecto sobre la g_m en hojas de plantas crecidas a 15°C versus 30°C (Warren et al., 2008).

Fotosíntesis y sus posibles limitantes en ambientes de alta montaña

La fotosíntesis en la alta montaña es interesante de estudiar debido a que estos ambientes son complejos desde el punto de vista climático, y presentan distintas condiciones que podrían ser adversas para las plantas (Korner 2021). Por ejemplo, a mayores elevaciones, existe una menor presión parcial de gases, lo cual puede afectar positiva o negativamente la fotosíntesis. A menor presión parcial de gases ocurre un aumento de las tasas de difusión, por lo que el CO_2 se mueve más rápidamente por el medio gaseoso hasta los espacios intercelulares aéreos de las células del mesófilo, esto afectaría positivamente la fotosíntesis. Por otro lado, existe también menor presión parcial de CO_2, lo cual resulta en una menor disponibilidad de este gas para la asimilación. También, la menor presión parcial de O_2 genera una disminución de la actividad oxigenasa de la RuBisCO, lo cual disminuye la competencia con la actividad carboxilasa, beneficiando la fotosíntesis. Wang y colaboradores (Wang et al. 2017) realizaron un análisis teórico sobre estas consideraciones, incluyendo los efectos de la baja temperatura que ocurre a medida que aumenta la elevación, concluyendo que la acción conjunta de todas estas variables debería resultar en una pequeña disminución en las tasas finales de fotosíntesis.

De acuerdo con lo anterior, se podría esperar que las tasas de fotosíntesis fuesen menores a las presentes en ambientes más benignos, pero la evidencia parece indicar que esto no es siempre así (Fan et al. 2011; Gratani et al. 2012; Guan et al. 2018; Gurevitch 1992; Korner & Diemer 1987; Kostopoulou & Karatassiou 2016; Ma et al. 2015; Shi et al. 2015; Vats & Kumar 2009). El hecho de que las tasas de fotosíntesis no sean necesariamente menores a mayor elevación podría explicarse con el hecho de que las plantas de alta montaña presenten características particulares acordes a su ambiente, las cuales les permiten alcanzar

tasas de fotosíntesis comparables a las de baja elevación. Una de estas características sería una alta eficiencia en la asimilación de CO_2. Körner y Diemer (1987) muestran cómo las plantas de distribución exclusivamente alpina poseen mayores pendientes en las curvas de fotosíntesis versus concentración interna de CO_2 comparado con plantas de menores elevaciones, característica que se asociaría a una alta velocidad de carboxilación de las plantas de mayor elevación (Gurevitch, 1992). También, Guan y colaboradores (2018) encontraron mayores velocidades máximas de carboxilación en plantas de mayor altitud comparado con plantas de altitudes intermedias, lo cual podría indicar una gran capacidad bioquímica de las plantas de grandes elevaciones, y por ende una menor limitación por procesos bioquímicos de asimilación de carbono.

Por otro lado, las características anatómicas de las hojas de zonas altas podrían tener una importante incidencia sobre las tasas de fotosíntesis. Diversos trabajos han mostrado la existencia de un patrón de hojas con menor proporción entre el área foliar y la masa de la hoja (SLA) a mayor elevación (Kao & Chang 2001; Kogami et al. 2001; Korner & Diemer 1987; Ma et al. 2015), esto significa que las hojas son más densas y/o más gruesas en zonas altas, lo cual podría deberse a una mayor inversión en pared celular a medida que aumenta la altitud, patrón que ha sido observado en estudios histoquímicos con la hierba de alta montaña *P. saundersiana* (Ma et al. 2015). Esta inversión en pared celular sería beneficiosa para las plantas al otorgar mayor resistencia y soporte al tejido a cambio de una menor tasa de fotosíntesis y difusión de CO_2 (Onoda et al. 2017). Debido a esto la conductancia del mesófilo podría ser una importante limitación a la fotosíntesis de las hojas presentes en zonas alpinas y aumentar con la elevación.

El caso de los Andes de Chile central

Chile central es una de las cinco zonas (junto a California, Sur de Sudáfrica, Sudoeste de Australia y la cuenca del mar mediterráneo) que poseen un clima de tipo mediterráneo, caracterizado por veranos largos, calurosos y secos (di Castri & Hajek 1976). Eso produce que en la cordillera de los Andes de Chile central la

temporada de crecimiento de las plantas de alta montaña, a diferencia de otras montañas en el mundo (e.g. los Alpes), sea más seca. Las zonas de menor elevación (alrededor de los 2500 m s.n.m.) de los Andes de Chile central no suelen tener precipitaciones en verano, lo cual resulta en una disminución del potencial hídrico del suelo a medida que avanza la temporada de crecimiento (Cavieres et al. 2006). En contraste, producto de precipitaciones de origen convectivo, las zonas de mayor elevación (alrededor de los 3600 m s.n.m.) suelen recibir precipitaciones durante la época estival (Viale & Garreaud 2014). La sequía de las zonas de menor elevación produce un ambiente adverso para las plantas, lo cual contrasta con el patrón descrito mundialmente de mayor adversidad ambiental a mayores elevaciones. Esta adversidad hídrica a bajas elevaciones se ve reflejada por ejemplo en la gran importancia de las interacciones de facilitación que realizan las plantas en cojín a altas y bajas elevaciones en los Andes (Cavieres et al. 2006) en contraposición al patrón descrito mundialmente donde las interacciones de facilitación aumentan con la elevación (Callaway et al. 2002). Los mayores niveles de sequía que ocurren a menores elevaciones generan también una serie de diferencias altitudinales en las plantas como los encontrados en *Phacelia secunda*. Para esta especie, Sanfuentes y colaboradores (2012) mostraron como a menor elevación un aumento de la temperatura a la que están sometidas las plantas produce menores tasas de fotosíntesis. Interesantemente, cuando las plantas eran sometidas a riego artificial, estas tasas de fotosíntesis eran recuperadas (Hernández et al., 2015). Se ha observado también que la adversidad hídrica produce diferencias altitudinales en los mecanismos de fotoprotección. Esto fue demostrado por Hernández y colaboradores (2015) al observar cómo a menores elevaciones la reacción de Mehler, fotorrespiración y transporte cíclico de electrones eran mayores, pero disminuían cuando las plantas eran irrigadas artificialmente. Debido a esto se esperaría que las plantas de menores elevaciones ajusten también su g_m como se ha observado en otras zonas no montañosas Mediterraneas (Limousin et al. 2010; Misson et al 2010). En estos trabajos se observó como las mayores restricciones hídricas del árbol *Qercus ilex* resultaron también en menores tasas de fotosíntesis, asociado a menores valores de g_m acompañado por Jmax, Vcmax y g_s.

Vegetación azonal

En contraposición a los altos déficits hídricos a los que están sujetas la gran parte de las plantas en la cordillera de los Andes de Chile central (vegetación zonal), existe también vegetación de tipo azonal (Fig. 2). Este tipo de vegetación se denomina así ya que está asociada a factores locales como la presencia permanente de humedad o anegamiento constante debido al agua de las capas freáticas, proveniente del derretimiento de nieve y glaciares (Ahumada & Faundez 2007). La vegetación azonal de los Andes de Chile central puede denominarse en sentido general como bofedal (Squeo et al., 2006). Estos bofedales existen en los Andes de Perú, Bolivia, Argentina y Chile, desarrollándose en los pisos vegetacionales más elevados dentro del rango de distribución de las plantas vasculares. En las distintas elevaciones donde crecen y bajo condiciones apropiadas, las plantas de los bofedales forman coberturas muy compactas, densas y en forma de cojín. Los brotes de las juncáceas predominantes en estos ecosistemas pueden crecer indefinidamente desde la parte terminal del tallo, mientras que las partes inferiores van muriendo paulatinamente formando turba en la parte inferior de la planta. En buenas condiciones hídricas y con anoxia, la turba formada puede ser de varios metros de espesor y funciona como un controlador del escurrimiento de agua hacia menores elevaciones.

Fig 2. Paisaje con vegetación zonal y azonal. La imagen muestra como la vegetación azonal crece en entornos con gran presencia de agua.

En estos ecosistemas no solo crecen juncáceas, sino que también existen otras plantas que dan lugar a ensamblajes de especies dónde las familias predominantes son Cyperaceas, Asteraceas, Plantaginaceas y Poaceas (Ruthsatz 2012). La composición de especies de estos ensamblajes está determinada por la humedad del sitio y salinidad del agua que entra en el humedal, así como también por la elevación del sitio en que crecen (Ruthsatz 2012). Por otro lado, el desarrollo de la vegetación azonal parece estar controlado principalmente por unos pocos factores ecológicos, entre los que destacan: 1) la cantidad de agua y su disponibilidad durante la estación. Y 2) las temperaturas ambientales favorables (Squeo et al. 2006), los cuales podrían ser mayores determinantes de la fotosíntesis de las plantas de alta montaña en general. Las plantas azonales podrían utilizarse como un experimento natural para contrastar en terreno los efectos de la disponibilidad hídrica a lo largo del gradiente altitudinal ya que estarían sujetas a los efectos de la disminución de las temperaturas, y otros factores ambientales, a medida que aumenta la elevación, pero no serían afectadas por la falta de agua, que van sufriendo el resto de las plantas zonales durante el verano.

En esta tesis se propone que la conductancia del mesófilo será una de las mayores limitaciones a la fotosíntesis de las plantas de alta montaña de los Andes de Chile central debido al efecto de las bajas temperaturas a mayor elevación y el déficit hídrico a menor elevación. Específicamente, esta condición de baja temperatura y limitados recursos hídricos, tendrían efectos sobre la anatomía foliar, aumentando la resistencia al flujo de CO_2 para la fotosíntesis. Por otro lado, las plantas de los sistemas azonales no estarán sujetas a restricciones hídricas y por ende no estarían sujetas a los cambios que afectarían a las plantas zonales. En este trabajo se utilizaron las especies zonales *Phacelia secunda* y *Bromus setifolius*, junto con las especies azonales *Colobanthus quitensis, Oxychloe andina y Plantago barbata* (Fig. 3).

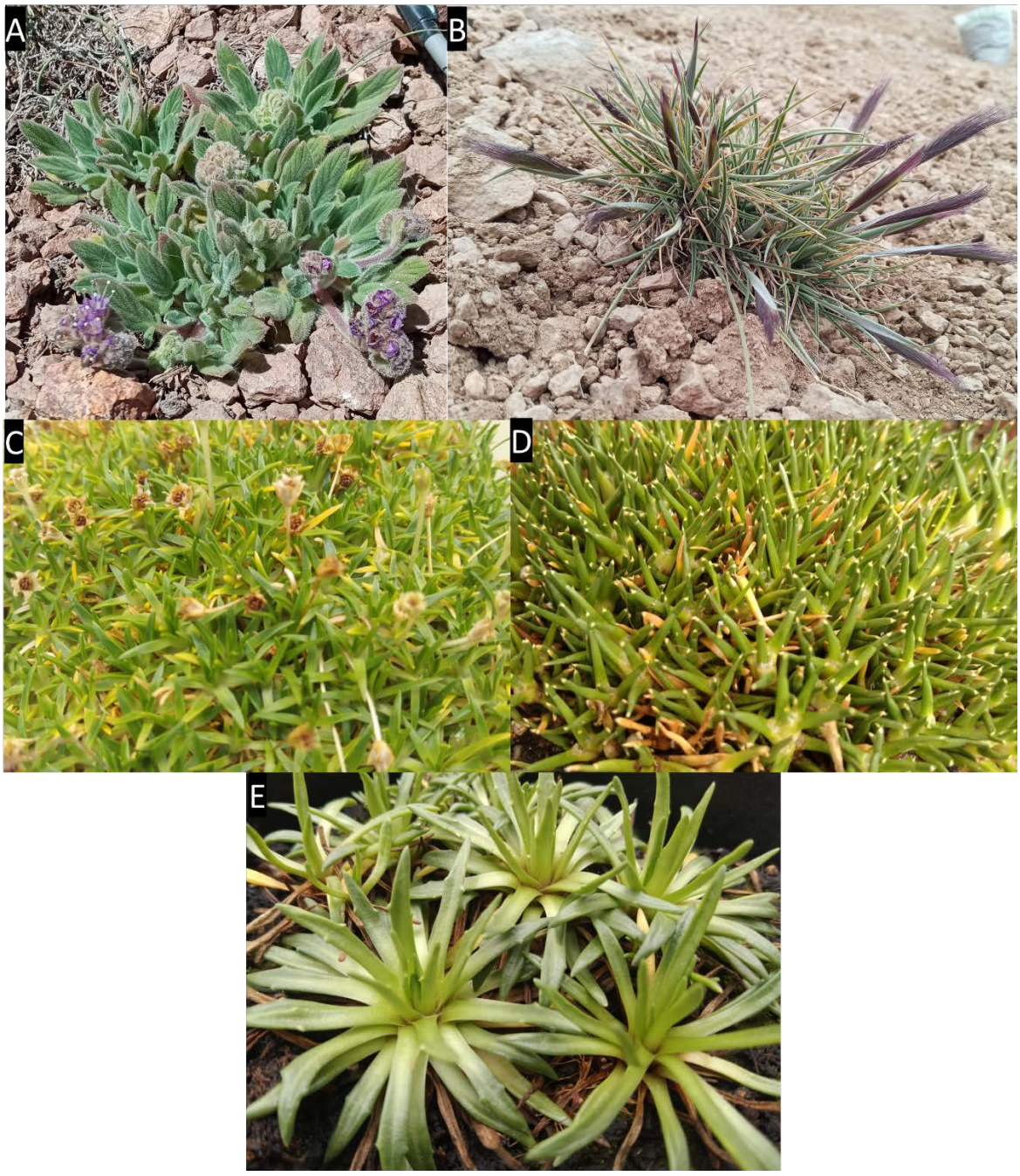

Fig. 3. Especies estudiadas. La imagen muestra las especies zonales estudiadas *Phacelia secunda* (A) y *Bromus setifoluis* (B), junto a las especies azonales *Colobanthus quitensis (C)*, *Oxychloe andina* (D) y *Plantago barbata (E)*.

Hipótesis

Las menores temperaturas y disponibilidad hídrica aumentan el LMA, lo que disminuye la gm y la fotosíntesis de plantas alto-Andinas de Chile central. Debido a esto, plantas zonales tienen alto LMA y baja g_m a mayor y menor elevación. Mientras que plantas azonales aumentan su LMA y disminuyen su g_m a mayor elevación.

Los cambios de g_m ante distintas condiciones ambientales se deben a cambios anatómicos como el grosor de la pared celular y la disposición de los cloroplastos.

La temperatura es el factor que impulsa las tasas fotosintéticas más bajas en las plantas de gran altitud y que el mecanismo subyacente a las variaciones en A_N asociadas a la temperatura involucra cambios en el LMA y, en consecuencia, en la g_m.

Objetivo general

Determinar las características y limitantes fotosintéticas de plantas zonales y azonales de los Andes de Chile central en un rango altitudinal durante la temporada de crecimiento, así como el efecto de la temperatura de crecimiento sobre estos parámetros.

Objetivos específicos

Determinar las tasas máximas de fotosíntesis de plantas zonales y azonales de los Andes de Chile central en un gradiente altitudinal y durante la temporada de crecimiento.

Estimar la partición de las limitaciones a la fotosíntesis de plantas zonales y azonales de los Andes de Chile central en un gradiente altitudinal y durante la temporada de crecimiento.

Evaluar los determinantes micro anatómicos de la g_m de plantas zonales y azonales en el gradiente altitudinal.

Evaluar el efecto de la temperatura sobre la conductancia del mesófilo y la fotosíntesis en plantas zonales y azonales de los Andes de Chile central.

Capitulo 1

Mesophyll conductance limits photosynthesis and relates to anatomical traits in high-elevation plants in the Andes

Running title: Photosynthetic limitations in high-elevation Andean plants

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Abstract

Plants face harsher conditions with increasing elevation, including shorter growing seasons, lower temperatures, and reduced gas pressure. This often leads to increased leaf mass per area, suggesting greater limitation to photosynthesis due to decreased mesophyll conductance. However, some species maintain consistent photosynthetic rates at higher elevations, suggesting compensatory mechanisms. In the central Chile Andes, high-elevation habitats present cold temperatures with no soil moisture deficits, whereas low-elevations experience warm temperatures and summer droughts. Zonal plants adapt to these changes, whereas azonal plants grow near water sources and avoid drought. We assessed how elevation affects photosynthesis and its limitations in these plant-types, together with the role of leaf internal anatomy. This was done with gas exchange and chlorophyll fluorescence analyses, along with measurements of leaf inner structure, on zonal and azonal species growing at 2600 and 3550 m a.s.l. Results showed that whilst photosynthesis decreased with elevation in azonal plants, zonal plants showed no change, with mesophyll conductance being a primary limitation, influenced by chloroplast arrangement rather than that cell wall thickness. This affects carbon acquisition in high-elevation plants due to low gas pressure and light availability.

Keywords: alpine plants, chloroplast, elevation, LMA, mesophyll conductance, photosynthesis.

Abbreviations

A_N = Net photosynthetic rate, ETR = Electron transport rate, g_m = Mesophyll conductance, g_s = Stomatal conductance, l_{bq} = Biochemical limitation, LD = Leaf density, LMA = Leaf mass per area, l_{mc} = Mesophyll limitation, l_s = Stomatal limitation, LT = Leaf thickness, S_c/S = Surface of chloroplasts exposed to intercellular air spaces per area, S_c/S_m = Surface of chloroplasts per mesophyll exposed to

intercellular air spaces, SD = stomatal density, S_m/S = Surface of mesophyll exposed to intercellular air spaces per area, T_{cw} = Cell wall thickness.

Introduction

For photosynthesis to occur, CO_2 must enter the leaf through the stomata and be transported through the mesophyll to the carboxylation sites in the chloroplast where it is fixed by the RuBisCO. Three major limitations have been described in this CO_2 voyage: (1) the stomatal limitations (l_s), related to the openness of stomata. This limitation becomes more significant when stomata are closed, hence generating low stomatal conductance (g_s); (2) Mesophyll limitations (l_{mc}), which occur when the transport of CO_2 through the leaf mesophyll (g_m) is the primary barrier to CO_2 transfer to the carboxylation site. This is attributed mainly to anatomical traits like cell wall thickness (T_{cw}), chloroplasts arrangement and mesophyll porosity (Evans, 2021), as well as to non-anatomical traits like aquaporins and carbonic anhydrase activity (Flexas et al., 2006; Price et al., 1994; Williams et al., 1996). And finally (3) biochemical limitations (l_{bq}), which occur when the biochemical capacity is insufficient to fix all the CO_2 entering the chloroplast, determined by the maximum RuBisCO carboxylation rate, the maximum capacity of electron transport and the triose phosphate utilization rate (Gago et al., 2019). The balance between g_s, g_m and the biochemical capacity of the leaf determines the relative importance of each limitation, which has been observed to vary in response to different environmental conditions such as light, temperature, water, or nutrient availability (Niinemets et al., 2009; Gago et al., 2020) as well as along the evolutionary transitions of terrestrial plants (Carriquí et al., 2020; 2019; Veromann-Jürgenson et al., 2017; 2020).

Plants inhabiting wide elevational gradients in mountain habitats are exposed to significant changes in some environmental conditions (Körner, 2021). For instance, as elevation increases, temperatures, partial pressure of gases, solar radiation, duration of the growing season and soil stability decrease (Körner, 2021). Despite the increase in environmental severity with elevation, a commonly observed pattern is that there are no significant differences in the photosynthetic rate of plant species inhabiting different elevations (Fan et al., 2011; Gratani et al., 2012; Guan

et al., 2018; Gurevitch, 1992; Körner and Diemer, 1987; Kostopoulou and Karatassiou, 2016; Ma et al., 2015; Shi et al., 2015; Vats and Kumar, 2009; Wang et al., 2017). This pattern has been explained by compensatory mechanisms at the leaf level that allow to achieve similar photosynthetic rates despite the environmental changes with elevation. For instance, the synthesis of larger amounts of proteins, including RuBisCO or isoforms that perform better at low temperatures has been suggested as a mechanism underlying higher maximum velocity of carboxylation with elevation (Huner and Macdowall, 1979; Yamori et al., 2006), and several studies have shown increases in biochemical parameters with elevation (Mehta et al., 2024; Peng et al., 2020; Körner et al.,1987; Guan et al., 2018). Therefore, current evidence suggests that biochemical limitations might not change with elevation despite the lower CO_2 partial pressure and temperatures characterizing high-elevation habitats.

Regarding diffusive limitations, it has been shown that g_s tends to increase with elevation. This can be attributed to the lower soil water restrictions occurring at higher elevations (Ma et al., 2015; Gurevitch et al., 1992; Shi et al., 2015). Additionally, as elevation increases, the water vapor pressure inside the leaf remains constant, while the partial pressure of vapor in the air decreases, assuming a constant molar fraction. This results in an increased delta for vapor pressure between the leaf and the surrounding air, and therefore higher g_s (Wang et al., 2017). However, elevational decreases in g_s, have also been observed in some alpine plants (Shi et al., 2015) and some herbs from more mesic environments (Kogami et al., 2001; Shi et al., 2006), which increases the diffusive limitations associated with stomata. Anatomical traits, such as stomatal density (SD) have also been used to infer stomatal limitations (Paridari et al., 2013). One hypothesis state that SD increases with elevation to compensate for the reduced carbon gain associated with the lower CO_2 partial pressure (Kouenberg et al., 2007; Woodward et al., 2001). However, as the elevation increases, both positive and negative variations in SD have been reported (Körner et al., 1986; 1983; Paridari et al., 2013). A commonly observed pattern along elevational gradients is the decrease in leaf size with smaller and more tightly packed cells resulting in greater leaf mass area (LMA) at higher elevations (Kao and Chang, 2001; Kogami et al., 2001; Körner and Diemer, 1987;

Ma et al., 2015). As higher LMA is typically due to enhanced leaf density (LD) and T_{cw} (Niinemets et al., 2009), this may result in lower g_m (Terashima et al., 2011; Onoda et al., 2017; Tomás et al., 2013), suggesting greater mesophyll limitation to photosynthesis at high elevation. However, few studies have addressed g_m changes with elevation, and no general trends have been found so far. For example, Guo et al. (2023) evaluated elevational changes in g_m in two conifer species along an elevation gradient from 2500 to 3200 m a.s.l. showing low l_{mc} at all elevations. Other studies have shown either increases or decreases in g_m with elevation (Kogami et al., 2001; Shi et al., 2015) but no assessments on its overall limitation to photosynthesis compared to stomatal and biochemical limitations were reported. The mechanisms that regulate g_m can vary in response to environmental changes, such as variations in light, water, or nutrient availability (Gago et al., 2020). Parameters such as the surface of chloroplast exposed to intercellular air spaces (S_c/S), the surface of mesophyll exposed to intercellular air spaces (S_m/S), the T_{cw}, chloroplast length and thickness have been found to change in other environments with similar adverse conditions as those present in high-elevation habitats (Tosens et al., 2012; Sáez et al., 2018; Flexas et al., 2008), which could result in significant changes in mesophyll limitations. However, the importance of this limitation on the photosynthetic rates of high-elevation plants remains poorly explored.

The high elevation habitats of the Andes of central Chile are characterized by an alpine climate with a strong influence of the Mediterranean-type climate prevailing at low elevations (Cavieres et al., 2006). Thus, elevational gradients in this zone runs from warm low-elevation habitats that face water deficits throughout the growing season to cold high-elevation habitats with no soil moisture shortages during the growing season (Cavieres et al., 2006; 2007; Sanfuentes et al., 2012; Hernández-Fuentes et al., 2015). It is well known that low soil water availability affects photosynthesis. Although it is generally observed that plants exposed to drought close stomata to avoid water losses in detriment of photosynthesis (Chaves, 1991; Flexas et al., 2004), the effects on g_m are not clear yet. While some studies have shown that g_m decreases with drought (e.g. Gallé et al.; 2009; Cano et al., 2013) others have found no changes (Bunce, 2009; Galmés et al., 2007; Hommel et al.,

2014; Warren et al., 2011). However, when the balance of photosynthetic limitations was evaluated, the species studied showed lower g_m and g_s, and an increase in the diffusive limitations when plants were exposed to drought (Flexas et al., 2009; Galle et al., 2009; Galmés et al., 2007). Drought induced changes in g_m were mediated by changes in anatomical parameters such as S_c/S, S_m/S, mesophyll porosity and T_{cw} (Tosens et al., 2012). Moreover, under water stress, the biochemical limitations appear later when g_s and g_m are already low (Bota et al., 2004; Ahrar et al., 2017).

Along the different elevations of the central Chile Andes, it is also possible to find peatbog habitats (bofedales) that develop in groundwater outcrops and/or runoff from glacier melt (Squeo et al., 2006) that maintain soils saturated with water throughout the entire growing season. Therefore, at lower elevations, plants inhabiting bofedales (azonal plants) do not experience the soil water limitations that affect other plant types, namely the zonal vegetation. This combination of conditions, where zonal plants experience differences in soil moisture and growth temperatures at contrasting elevations (low *vs*. high), whilst azonal plants only experience temperature differences, constitutes an ideal natural laboratory to study photosynthetic patterns and the magnitude of elevational changes of l_{bq}, l_s and l_{mc} to photosynthesis. This can provide valuable insights into the importance of leaf traits and their impact on plant performance at different elevations and in different plant types (zonal and azonal). In particular, we hypothesized that photosynthesis does not show altitudinal differences due to compensatory mechanisms like higher SD in both zonal and azonal plants. For zonal plants the LMA and the mesophyll limitation will not change with elevation as both high and low elevation habitats present harsh environmental conditions such as low temperatures and low soil moisture, respectively. In contrast, for azonal plants, we expect higher LMA and mesophyll limitation at high elevation. To test these hypotheses, we studied the photosynthesis and photosynthetic limitations, measured through leaf gas exchange and chlorophyll fluorescence, together with different anatomical and structural traits on two zonal species and three azonal species growing at two contrasting elevations (2600 and 3550 m a.s.l.) in the central Chile Andes.

Materials and methods

Study sites

The study was conducted in the central Chile Andes, at 50 km east of Santiago. Two sites at two different elevations were chosen: 2600 and 3550 m a.s.l. At both elevations two plant types were studied: the azonal species *Plantago barbata* (G. Frost Plantaginaceae), *Oxychloe andina* (Phil. Juncaceae) and *Colobanthus quitensis* (Kunth Caryophyllaceae) and the zonal species *Bromus setifolius* (J. Presl Poaceae) and *Phacelia secunda* (J.F. Gmel Boraginaceae), the species were selected according to two criteria: presence in both elevations and possibility to be measured with gas exchange device. As mentioned, azonal and zonal vegetation are characterized by different soil moisture availability, while azonal plants have a full water supply during the growing season, zonal plants depend on the few precipitation events during the growing season (Squeo et al., 2006).

At lower elevation the growing season usually starts in October and ends in April. The annual precipitation is around 700 mm, predominantly falling as snow in winter (Santibáñez and Uribe, 1990), and the mean air temperature is around 10.7°C (Hernandez-Fuentes et al., 2015). The mean gravimetric soil water contents at the site of *Phacelia secunda* and *Bromus setifolius* was 0.040 g g^{-1} and 0.035 g g^{-1}, respectively. The higher elevation site presents a 4-month growing season (from December to April), and it is characterized by an average precipitation of 943 mm, falling as snow in winter and occasionally as rain, hail, and snow during the growing season. At this site, mean temperature is around 8.2°C (Hernandez-Fuentes et al., 2015), the mean gravimetric water content for zonal plants was 0.095 g g^{-1} and like in the lower elevation, the soil in peatland areas was fully saturated with water. Gas exchange, chlorophyll fluorescence, and anatomy were measured early in the growing season between December and January 2021-2022.

Gas exchange and chlorophyll fluorescence

Leaf gas exchange was determined simultaneously with chlorophyll fluorescence using a LI-6400XT gas exchange system (LI-COR Inc., Lincoln, NE, USA) with an integrated fluorescence chamber (Li-6400-40; LI-COR Inc.). Measurements were taken on a single leaf or groups of leaves, aiming to cover the entire area of the IRGA chamber without overlapping, following the procedure described in Sáez et al. (2017). The leaf area was determined by taking a photograph and measuring the area within the chamber using the ImageJ software (Wayne Rasband/NIH, Bethesda, MD, USA). The conditions inside the leaf chamber consisted of photosynthetically active radiation (PAR) of 1500 µmol photons $m^{-2}s^{-1}$ for all species except for *C. quitensis* which was 1000 µmol photons $m^{-2}s^{-1}$, with a 10% of blue light, relative air humidity between 40% and 60%, and leaf temperature set at 15°C.

Light response curves were performed at different light intensities ranging from 30 to 130 µmol $m^{-2}s^{-1}$ under low oxygen concentration, following the method proposed by Bellasio (2016). The reduction of ambient oxygen concentration was achieved by connecting a nitrogen cylinder to the air inlet of the gas exchange system, replacing the ambient gas concentration with an inert one for the leaf. Corrections for CO_2 leakages of the leaf chamber were applied to all gas exchange measurements, as described in Flexas et al. (2007).

The quantum efficiency of photosystem II (PSII) was determined using the equation:

$$\phi PSII = \frac{(F'm - F's)}{F'm}$$

Where F'_s represents the steady-state fluorescence under constant light (PPFD 1500 µmol $m^{-2}s^{-1}$), and F'_m represents the maximum fluorescence obtained after a saturating light pulse of 8000 µmol $m^{-2}s^{-1}$ and 0.8 s of duration. As ϕPSII represents the number of electrons transferred per absorbed photon by PSII, the electron transport rate (ETR) can be calculated as ETR = φPSII · PPFD · αβ, where

PPFD is the photosynthetically active photon flux density, and αβ was estimated from the parameters obtained from the low-oxygen light response curves.

2.3 Estimation of mesophyll conductance

The mesophyll CO_2 conductance was calculated based on the combination of gas exchange and chlorophyll fluorescence, following the method described in Harley et al. (1992):

$$g_m = \frac{A_N}{C_i - \frac{(\Gamma(ETR + 8(A_N + R_n))}{(ETR - 4(A_N + R_l)))}}$$

A_N (net photosynthetic rate) and C_i were obtained from gas exchange measurements under saturating light conditions. The rate of non-photorespiratory CO_2 evolution under light conditions (R_l) was assumed to be half of the dark respiration (R_n). The CO_2 compensation point (Γ*) was calculated considering the altitudinal effects of changes in partial pressure of oxygen according to the following equation Γ*=0.5 O/($S_{c/o}$), where O is the partial pressure of oxygen and $S_{c/o}$ is the specificity factor of RuBisCO (Farquhar et al., 1980). The determination of g_m was used to calculate C_c.

Leaf mass per area, density and thickness

At each elevation, and for each study species eight to ten leaves were collected and scanned, and their areas measured with the ImageJ software (Wayne Rasband/NIH, Bethesda, MD, USA). Afterwards they were dried in oven at 70°C for 48 hours to measure their dry weight and calculate the LMA dividing mass by area. Leaf density was calculated dividing LMA by leaf thickness (LT) obtained from microscopy images.

Stomatal density

The anatomical characteristics of the stomata were measured in the middle leaf section on both the adaxial and abaxial faces. The leaves were collected at solar noon and immediately fixed in FAA (formalin-glacial acetic acid–70% ethanol). Afterward, plant material was boiled in 96% ethanol for 10 min and subsequently boiled again in an aqueous solution (1:1) of 96% ethanol and 5% sodium hydroxide for 10 min. The material was washed twice with distilled water, and then a 50% sodium hypochlorite solution was applied and allowed to stand for 12 min until the leaves became transparent. Once the material was rinsed, it was washed five times with distilled water and keptina 5% chloralhydrate solution for 10 min. For staining, a 20% solution of safranin in 96% ethanol was applied for 5 min, and 50% glycerin was applied to mount the samples. Samples were photographed with an optical microscope (CX, Olympus, Japan) and micrographs were analyzed using the ImageJ software (Wayne Rasband/NIH, Bethesda, MD, USA). Stomatal density was determined by averaging the number of stomata per unit of adaxial and abaxial epidermal area. As *C. quitensis* and *O. andina*, had stomata only in the border of the adaxial epidermis, the stomata present in the adaxial epidermis were weighted by the percentage of epidermis that presented stomata to calculate stomatal density.

Micro-anatomical leaf traits

At each elevation, for each study species, leaf samples were always collected at solar noon. The central portion of leaves were fixed in formaldehyde, acetic acid, and ethanol (FAA), and 3.7% glutaraldehyde, stored at 4°C and sent to the electron microscopy unit of the Universidad Austral de Chile where sample preparation and photography were carried out. After arrival, the samples were then washed with cacodylate buffer 0.1 M at 4°C. Post fixed with 1% osmium tetroxide for 2 h at 4°C. Afterwards the samples were washed with distilled water and dehydrated with increasing concentrations of ethanol 30, 50 and 70° for 45 min at 4°C, kept overnight

at 70° ethanol. The next day, we continued dehydration with ethanol 80, 96 and 100° for 45 min at 4°C, then the samples were put on acetone for 20 min and pre embed in acetone/spurr resin (3:1) overnight in agitation and ambient temperature. The next day, the samples were put acetone/resin (1:1) for 8 h and then in acetone/resin (1:3) overnight in agitation. The next morning, samples were put on pure resin for 24 h. Then, the samples were put on resin for 8 hours on a vacuum chamber. Later the samples were put on the silicon mold and polymerized at 70°C for 24-48 h. Finally, 250 nm slices were stained with toluidine blue. Once the mesophyll areas were selected 80 nm slices were stained with uranyl acetate 4% in methanol for 2 min and lead citrate for 5 min for transmission electron microscopy (EM1200 EXII; Japan Jeol Ltd, Tokyo, Japan). Six micrographs were randomly selected to measure micromorphological parameters; in each of these micrographs every cell was measured and averaged to obtain one value of each parameter and three values were taken for each species at each elevation. The parameters obtained were mesophyll porosity, thickness of the cell wall, distance between chloroplast and cell wall, chloroplast thickness, lateral distance between chloroplasts, S_m/S and S_c/S. 1000x magnification was used to measure parameters like S_c/S, while 10000x was used to measure mean cell wall thickness and chloroplast parameters. All the images were analyzed with ImageJ software (Wayne Rasband/NIH, Bethesda, MD, USA).

Quantitative analysis of photosynthetic limitations

To separate the relative control over A_N from limited stomatal conductance (l_s), mesophyll conductance (l_{mc}), and limited biochemical capacity (l_b) ($l_s+l_{mc}+l_b=1$), the quantitative analysis of limitations by Jones (1985), implemented according to Grassi and Magnani (2005) was used. The limitations of the different components were calculated as follows:

$$l_s = \frac{(g_{tot}/g_s)\delta A_N/\delta C_c}{g_{tot}+ \delta A_N/\delta C_c},$$

$$l_{mc} = \frac{(g_{tot}/g_m)\delta A_N/\delta C_c}{g_{tot} + \delta A_N/\delta C_c},$$

$$l_{bq} = \frac{g_{tot}}{g_{tot} + \delta A_N/\delta C_c},$$

where g_s is the stomatal conductance, g_m is the mesophyll conductance according to Harley et al., (1992), and g_{tot} is the total conductance for CO_2 from the ambient air to the chloroplasts was calculated as:

$$g_{tot} = \frac{1}{(1/g_s + 1/g_m)}$$

Statistical analyses

Linear mixed effects models were used to evaluate the effects of elevation and plant type (azonal or zonal) on A_N, g_s, g_m, LMA, LD, LT, and limitations to photosynthesis. In these models, species identity was included as a random factor to account for the interdependence of observations from the same species. A_N and g_m were log-transformed to reduce deviation from normality. Individual observations of these variables were back transformed to ease the visualization of the results (Fig. 1). Models consisting of full combinations of fixed factors and interaction terms were tested, the model quality being assessed by Akaike information criterion (AIC) values. Here, we reported the significance values for the best model for each plant response (Table 1.1). Normality of residuals was checked visually through histograms of normalized residuals, and by conducting Shapiro-Wilk normality tests. To check for homoscedasticity, the residuals versus fitted values were plotted. For each model, the overall variance explained (conditional R2) was calculated using the package 'MuMIN'. P-values were obtained from F-statistics of type III sum of squares using the Satterthwaite approximation to estimate the denominator degrees of freedom. The mixed models were performed using the lme4 package in the R statistical software (v. 3.5.1; R Core Team 2023).

To assess elevational differences between limitations to photosynthesis of the same plant type we used a one-way ANOVA, where differences among limitations means were assessed by a posteriori Tukey test ($P < 0.05$). The relationships between photosynthetic variables and morphological or anatomical traits were tested using linear regression analyses. All these analyses were done in Past 4.07b.

Results

Net photosynthesis ranged from 3 to 28 mmol CO_2 $m^{-2}s^{-1}$ and did not differ between zonal and azonal plant species, nor between elevations. However, we found a significant effect of the interaction between plant type and elevation (Table 1.1) indicating that zonal and azonal plants respond differently to elevation (Fig. 1.1A). Electron transport rate ranges from 36 to 217 mmol $m^{-2}s^{-1}$ (Table S 1.1) and correlated with photosynthesis in both plant-type (Fig. S1.1). Similar trends were observed for g_m, where no differences were found between zonal and azonal species, nor between elevations, but the interaction between these factors was significant (Table 1.1) (Fig. 1.1C). In contrast, g_s did not differ between elevations or plant-type, nor did the interaction between these factors (Table 1.1, Fig. 1.1B). Despite these general trends, species-specific variations can be observed (Fig. S1.2-4). Mesophyll conductance correlated with A_N in azonal and zonal plants while g_s correlated with A_N only in zonal plants (Fig. 1.2).

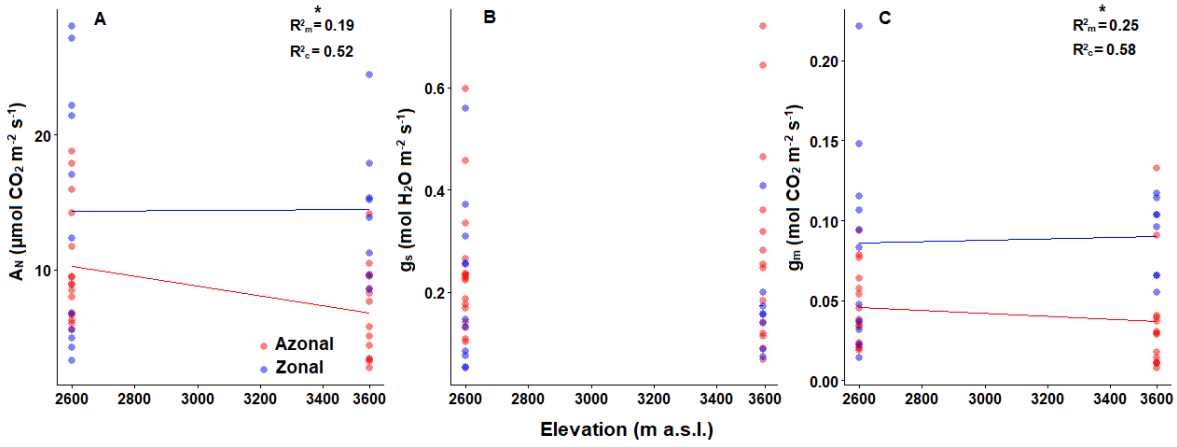

Fig. 1.1. Responses of net photosynthesis (A), stomatal conductance (B) and mesophyll conductance estimated by the Harley method (C) to elevation for zonal (blue) and azonal (red) plant species. Significant regression lines are based on model predictions (Table 1.1) between elevation and leaf traits at $p<0.05$ (*). Conditional (R^2_c) and marginal (R^2_m) coefficients of determination for mixed-effects models are shown.

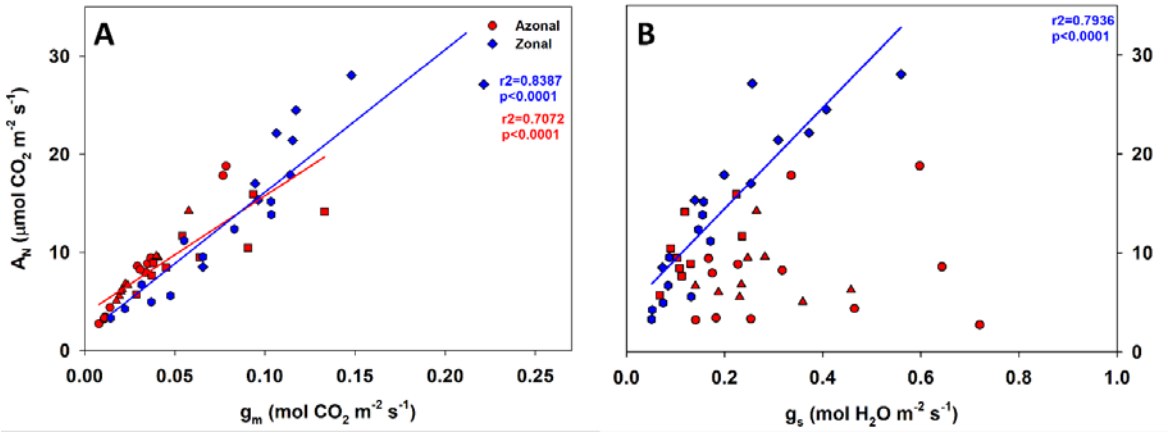

Fig. 1.2. Correlation between photosynthesis and diffusive components. The relationship between A_N with g_m estimated by the Harley method (A) and g_s (B) of five alpine species from different environments: Azonal (red) *Plantago barbata* (square)*, Colobanthus quitensis* (circle)*, Oxychloe andina* (triangle)*.* Zonal (blue): *Phacelia secunda* (diamond) and *Bromus setifolius* (hexagon). The straight lines correspond to a statistically significant linear regression.

Overall, LMA differed between plant types ranging between 38 to 144 g m^{-2}, being higher in zonal plants compared to azonal plants. Although no differences were found between elevations, the interaction between plant type and elevation was significant indicating that the responses to elevation differed between plant types (Fig. 1.3A). LD was higher in zonal plants compared to azonal plants, and an interaction effect between the plant type and elevation was found (Fig. 1.3B). Conversely, LT was higher in azonal plants compared to zonal plants (Fig. 1.3C). Species-specific variations of these traits are shown in Fig. S1.5-7.

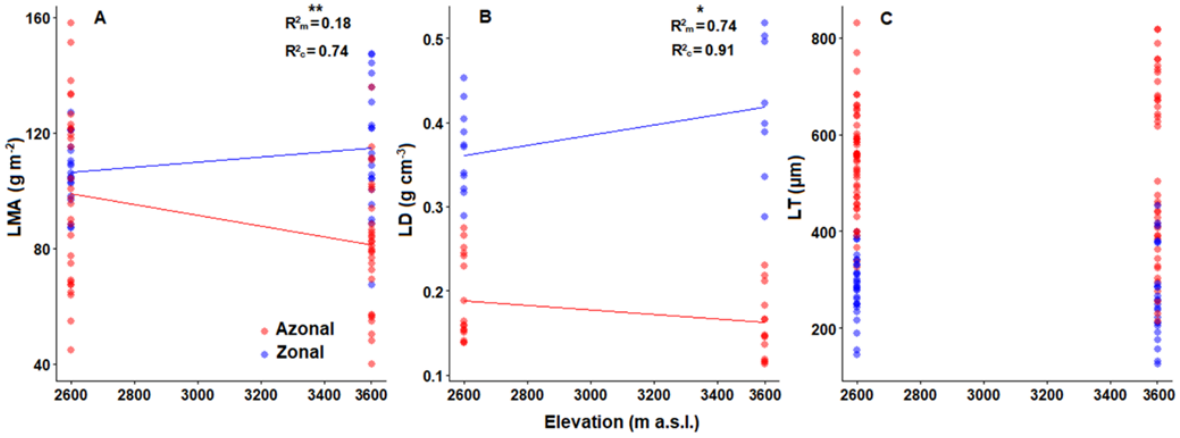

Fig. 1.3. Responses of leaf mass area (A), leaf density (B) and leaf thickness (C) to elevation for zonal (blue) and azonal (red) plant species. Regression lines are based on model predictions (Table 1.1) between elevation and leaf traits at $p<0.05$ (*) and $p<0.001$ (**). Conditional (R^2_c) and marginal (R^2_m) coefficients of determination for mixed-effects models are shown.

The stomatal limitation l_s showed a significant effect of the interaction between plant type and elevation (Fig. 1.4A). The mesophyll and biochemical limitations were not different between elevations nor between plant-types when considering all individuals together and showed species-specific variation within plant types (Fig. S1.8-10). In addition, the interaction between elevation and plant-type was not significant (Fig. 1.4B,C).

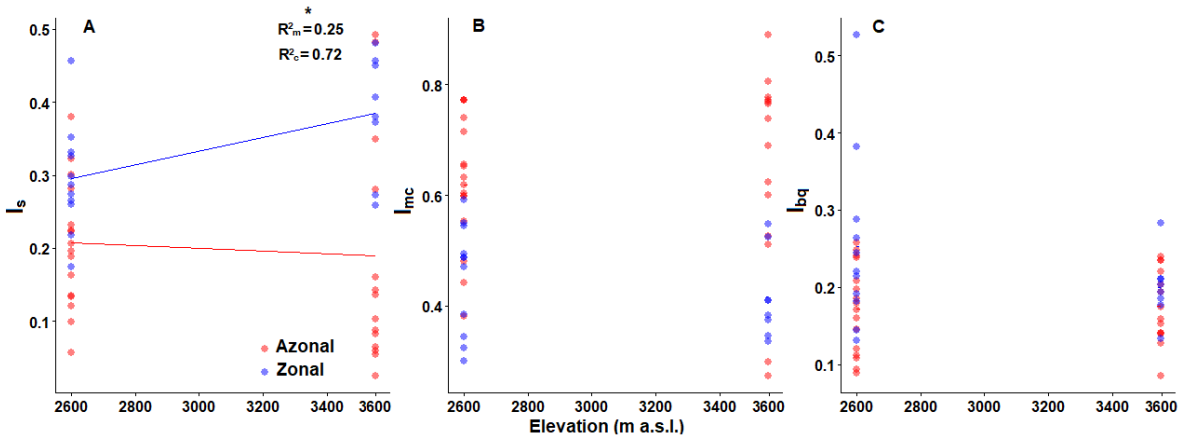

Fig. 1.4. Responses of stomatal limitation (A), mesophyll conductance limitation (B) and biochemical limitation (C) to elevation for zonal (blue) and azonal (red) plant species. Regression lines are based on model predictions (Table 1.1) between elevation and leaf traits at p<0.05 (*). Conditional (R^2_c) and marginal (R^2_m) coefficients of determination for mixed-effects models are shown.

Table 1.1. Effects of elevation and plant-type (zonal vs azonal) in different photosynthetic and morphological traits of high-elevation species of the central Chile Andes. Significant p values (< 0.05) from the GLM are presented in bold letters.

	Fixed effects								
	Elevation			Plant-type			Elevation x Plant type		
Response traits	d.f	F	P	d.f.	F	P	d.f.	F	P
A_N (log)	42.18	0.61	0.43	39.79	3.12	0.08	42.18	**5.89**	**0.01**
g_m(log)	42.17	0.12	0.72	38.45	1.60	0.21	42.17	**4.54**	**0.03**
g_s	44.35	0.01	0.91	49.00	0.47	0.49	44.35	0.90	0.34
LMA	92.00	2.21	0.14	11.40	**5.03**	**0.04**	92.00	**17.71**	**0.00**
LD	42.10	0.02	0.87	32.28	0.10	0.74	42.10	**7.94**	**0.01**

LT	141.32	0.50	0.48	5.05	**11.08**	**0.02**			
l_s	42.08	2.47	0.12	24.78	1.43	0.24	42.08	**5.52**	**0.02**
l_{mc}	42.08	0.17	0.67	25.60	0.01	0.90	42.08	0.82	0.36
l_{bq}	43.03	1.48	0.22	44.64	3.12	0.08	43.03	1.87	0.17

When comparing the magnitude of the different limitations to photosynthesis, l_{mc} was statistically higher than l_s and l_{bq} in azonal plants regardless of elevation (Fig. 1.5 A,B). Instead, in zonal plants, l_{mc} was higher than l_s and l_{bq} only at low elevation, while at the high elevation site l_s and l_{mc} co-limited photosynthesis with a lesser contribution of the biochemical component (Fig. 1.5 C,D).

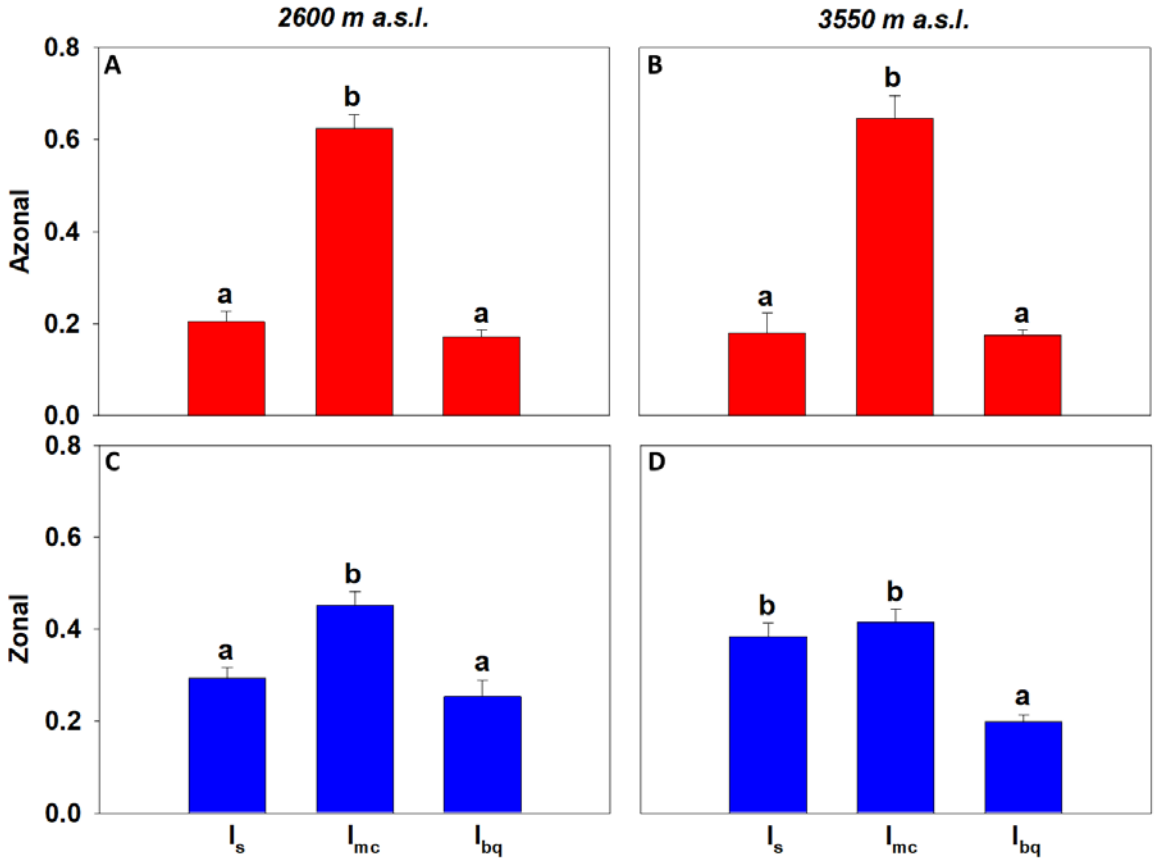

Fig. 1.5. Limitations to photosynthesis in azonal (A,B) and zonal (C,D) plants from 2600 m a.s.l. and 3550 m a.s.l. Lowercase letters indicate Tukey tests differences ($p<0.05$).

To search for the determinants of the high l_{mc} and low g_m values a correlation analysis of these parameters with morphological and anatomical leaf traits was used. Values of the different anatomical traits estimated are shown in Table S 1.2, and reference images from the low elevation chloroplasts and cell walls are shown in Fig. S1.11. When pooling all the samples together no correlation between g_m and LMA (Fig. 1.6A) was found, and a negative correlation was found between l_{mc} and LMA (Fig. 1.6E). T_{cw} did not correlate with g_m nor l_{mc} (Fig. 1.6B,F), but the parameter S_c/S_m correlated positively with g_m (Fig. 1.6C) and negatively with l_{mc} (Fig. 1.6G). In addition, SD had a positive correlation with l_{mc} (Fig. 1.6H).

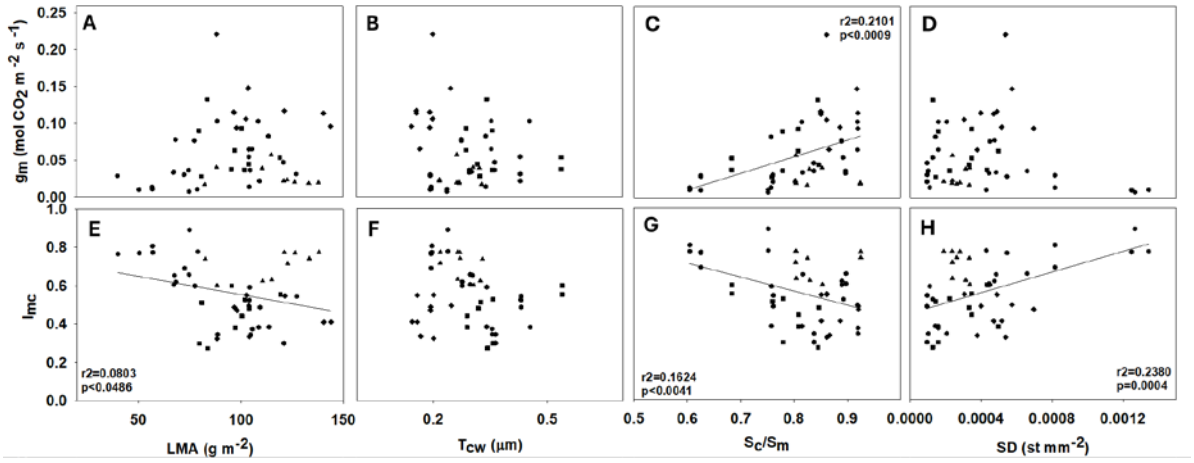

Fig. 1.6. Regression analysis between anatomical traits and mesophyll parameters for all species. The graphs show dot plot between leaf mass area (A), cell wall thickness (B), surface of mesophyll occupied by chloroplasts by surface of mesophyll exposed to intercellular air spaces (C) and stomatal density (D) mesophyll conductance estimated by the Harley method (g_m) and the same parameters with mesophyll limitation (l_{mc}) in panels E, F, G and H respectively. Black lines represent

the regression for all samples pooled, coefficient of determination and p values are shown in the graphs.

As zonal and azonal plant species have different characteristics, we evaluated these correlations on the two plant types separately. When separating zonal and azonal plants we found no correlation between g_m nor l_{mc} with LMA (Fig. S1.12A,E). T_{cw} correlated negatively with g_m in zonal plants and correlated positively with l_{mc} in azonal plants (Fig. S1.12 B,F). In zonal plants S_c/S_m correlated positively with g_m (Fig. S 1.12C) but showed no correlations with l_{mc} (Fig. S 1.12G). However, SD had a positive correlation with g_m in zonal plants and a negative correlation with azonal plants (Fig. S 1.12D). SD also had a positive relationship with l_{mc} in azonal plants (Fig. S 1.12H).

As plants from the low-elevation site have shown different responses compared to the high-elevation site, the correlation of these parameters was also evaluated by separating plants from high and low elevation sites. Then, when separating plants according to elevation we found that plants from the high-elevation site showed a positive correlation between g_m and LMA and a negative correlation between l_{mc} and LMA (Fig. S 1.13A,E). T_{cw} correlated negatively with g_m only in the low elevation site (Fig. S 1.13B). In the high-elevation site S_c/S_m correlated positively with g_m (Fig. S 1.13C) and negatively with l_{mc} (Fig. S 1.13G). Also, SD had a positive correlation with g_m in the low-elevation site and a negative correlation in the high-elevation site. SD also had a positive relationship with l_{mc} only in the high-elevation site (Fig. S 1.13H).

Discussion and conclusion

We hypothesized no altitudinal changes in photosynthesis regardless plant-type (azonal and zonal) in the central Chile Andes. Contrary to our expectations, we found that azonal plants showed lower photosynthesis at the high elevation site with no changes in stomatal density (Fig. S 1.14). Further, these plants decreased the LMA

with elevation. Decreases in photosynthesis with elevation have also been found in other studies supporting that despite the existence of some compensation mechanisms (i.e. increases in stomatal density, Körner 2021) the environmental changes occurring with elevation negatively affect photosynthesis (Ma et al., 2015; Gratani et al., 2014; Kao and Chang, 2001; Kogami et al., 2001). For instance, Ma et al. (2015) discussed that the elevational decreases in photosynthesis found in *Potentilla saundersiana* were explained by the lower temperatures experienced by these plants at high elevation (see also Gratani et al., 2014 for similar results). In our case the combination of the lower temperatures and LMA can explain the lower photosynthesis found in azonal plants. The lower LMA of these plants might be related to their growth form as these plants grow very close to each other forming dense and tightly knit carpets around cold water streams which may lead to competition for light. It is well known that lower light availability negatively affects LMA (Poorter et al., 2009), which in our case interacts with the lower temperatures, elevational decreases in CO_2 partial pressure and frequent overcast conditions decreasing LMA and photosynthesis in azonal plants. Indeed, when photosynthesis is expressed on a mass basis no elevation changes were observed (Fig. S 1.15) supporting the idea of an LMA dependent reduction of photosynthesis of azonal plants from high elevation.

Zonal plants responded differently, showing no changes in photosynthesis but higher LMA with elevation. In other studies (e.g. Gratani et al., 2014; Kao and Chang, 2001; Kogami et al., 2001) it has been shown that with elevation plants increased LMA but still showed lower photosynthetic rates than at lower elevations. Thus, this absence of elevational changes seems to be related with low photosynthetic rates at both elevations, which could be related to the lower soil moisture found at lower elevations (Cavieres et al. 2006) which has shown to restrict the photosynthesis and carbon gain dynamics in species inhabiting these sites (Hernández-Fuentes et al., 2015; Reyes-Bahamondes et al., 2021). It is known that low soil moisture affects photosynthesis through g_s (Chaves, 1991; Flexas et al., 2004). We found that stomatal conductance was associated with photosynthetic rate only in the zonal species (Fig. 1.2B) supporting the idea that the lower water availability of low

elevation habitats restrict photosynthesis generating adverse environments for plant carbon gain, similar to those found at high elevation but due to low temperatures and lower CO_2 partial pressure. Thus, our results show that elevational photosynthetic patterns may depend on plant-types associated to the soil moisture availability.

We found that the interaction between elevation and plant-type had a significant effect on the mesophyll conductance (Fig. 1.1C), resulting in a strong coupling between photosynthesis and g_m on both plant-types (Fig. 1.2A). This indicates that g_m is a strongest determinant of photosynthesis in the high-elevation plant species of the central Chile Andes assessed. The low values of g_m might explain the lack of correlation between g_s and A_N in azonal plants where the low water vapor pressure deficit around the leaves may force them to have high g_s to maintain the internal water flow as compensatory mechanisms to the notably low g_m. It has been shown that the photosynthesis of some high elevation plants is determined by fine-tuned stomatal and mesophyll conductance adjustments (Kogami et al., 2001) which is supported by our results in the central Chile Andes.

The importance of g_m on the photosynthesis of the high-elevation species studies is reinforced by the evaluation of the photosynthetic limitations (l_{mc}, l_{bq} and l_s) where l_{mc} was the highest for both azonal and zonal plants regardless elevation. The high l_{mc} values are due to the low l_s and l_{bq}, that might be related to high SD and biochemical capacity that some high-elevation species have shown (Körner et al., 1987; Peng et al., 2020). This may explain the negative correlation between l_{mc} and SD, further increasing the importance of l_{mc}. The mesophyll conductance limitation appears to be influenced by S_c/S_m, highlighting the role of chloroplast arrangement for photosynthesis (Sáez et al., 2017; Carriquí et al., 2019) and as far as we are aware this is among the first assessments for high-elevation plant species. All in all, our results suggest that g_m is a pivotal determinant of photosynthesis at high elevations, associated to the exposed mesophyll surface area occupied by chloroplasts and the proportionally low g_m compared to g_s. These traits seem to be more important than factors like LMA or T_{cw} in determining mesophyll conductance and mesophyll limitation in alpine plants from the central Chile Andes.

We expected to find no change in LMA and l_{mc} in zonal plants given the low soil moisture at lower elevation and low temperatures at high elevation, while for azonal plants it was thought to increase with elevation due to higher LMA and mesophyll limitations. Contrary to expectations, LMA values were higher in the high elevation zonal plants (Fig. 1.3A), concurring with the reported in Körner et al. (1987), Ma et al. (2015), Kogami et al. (2001), and Gratani et al. (2014). Nonetheless, no altitudinal differences were observed in l_{mc} for these plants (Fig. 1.4B), suggesting that l_{mc} is not totally dependent of LMA. In azonal plants, despite the lower temperatures of high elevation environments a lower LMA was found with no changes in l_{mc}, as previously mentioned, due to lower light availability. Thus, the correlation between g_m and LMA described in the foliar economic spectrum by Onoda et al. (2017) was not observed in our study (Fig. 1.6). These results underscore the importance of plant-types and species-specific effects of elevation on g_m, l_{mc} and LMA that can deviate from the expectations based on the leaf economic spectrum under certain environmental conditions.

It is worth to note that l_s for zonal and azonal species responded differently to elevation (Fig. 1.4C). While for azonal plants l_s remained similar at the two elevations, in zonal plants it showed an increase. The higher l_s could be explained by the greater difference in vapor pressure deficit between the leaf and environment at higher elevations (Wang et al., 2017) given the lower partial pressure of gases. Additionally, at higher elevations, greater stomatal opening to increase CO_2 intake (Kouenberg et al., 2007; Woodward et al., 2002), leads to lower leaf temperatures in high elevation environments where temperatures are already low. This interaction between optimal temperature and CO_2 uptake through stomata could help explain the increased stomatal limitation at higher elevations. Our results suggest that despite the low sensitivity of biochemical and mesophyll conductance limitations to elevational changes, stomatal conductance limitation may increase with elevation due to low partial pressure of CO_2 and temperature in zonal plants.

According to our knowledge, there are no studies that address the photosynthetic limitations in plants from high-elevation environments or demonstrate

that l_{mc} is indeed the primary photosynthetic limitation in these plants. This limitation was not primarily related to T_{cw} and LMA, as LMA seems to be diminished in azonal high elevation plants. Instead, l_{mc} appears to be a more important limitation due to chloroplast characteristics and the relatively high stomatal densities compared to the low values of g_m, along with the high biochemical capacity. Thus, this study provides an example where the relationship between g_m and LMA differs from that expected from the foliar economic spectrum and proposes some answers to the question of what environmental factors (i.e. low temperatures, low partial pressure of gases or soil moisture availability) could determine elevational changes in different plant traits related to photosynthesis.

Acknowledgements

We thank to the microscopy section from Universidad Austral (CMA), Ricardo Espinoza and Mauricio Avendaño, ECOBIOSIS Lab, RV and CR thanks their ANID doctoral scholarships.

Supplementary information

Table S 1.1. Electron transport rate (ETR) measured in species at 2600 and 3550 m a.s.l. in the Andes of central Chile. Significant differences (p value < 0.05) according to t or Mann-Whitney test are indicated with asterisk.

	ETR (umol m^{-2} s^{-1})		
Species	2600	3550	Dif.
B. setifolius	59.3	94.7	n.s
P. secunda	182.3	145.4	n.s
C. quitensis	124.8	78.9	*
O. andina	129.0	68.3	n.s.
P. barbata	106.1	95.3	n.s.

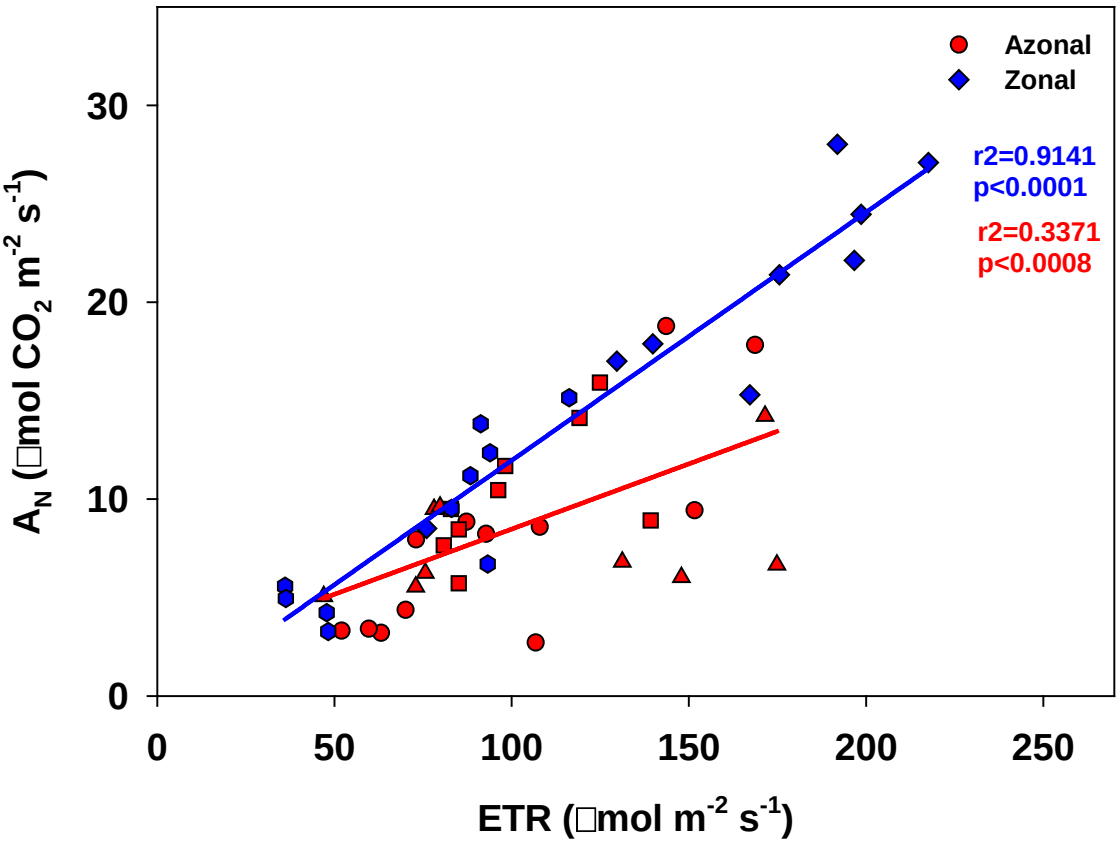

Fig. S 1.1. Correlation between electron transport rate and net photosynthesis. The relationship between ETR with A_N of five alpine species from different environments: Azonal (red) *Plantago barbata* (square), *Colobanthus quitensis* (circle), *Oxychloe andina* (triangle). Zonal (blue): *Phacelia secunda* (diamond) and *Bromus setifolius* (hexagon). The straight lines correspond to a statistically significant linear regression.

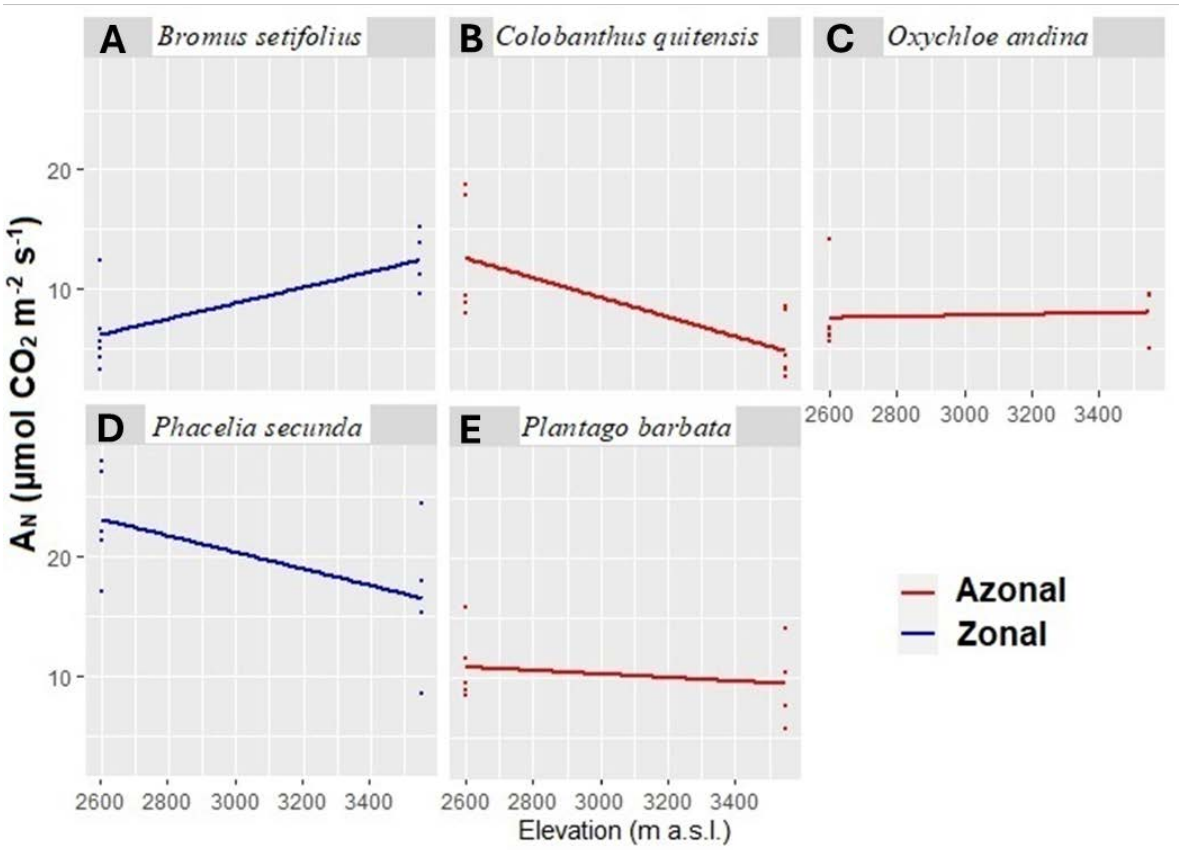

Fig. S 1.2. Dot plot and regression lines of net photosynthesis at different elevations. The graph shows photosynthesis from zonal (blue) *Bromus setifolius* (A) and *Phacelia secunda* (D), and azonal (Red) *Colobanthus quitensis* (B), *Plantago barbata* (C) and *Oxychloe andina* (E) plants from 2600 and 3550 m. a s l.

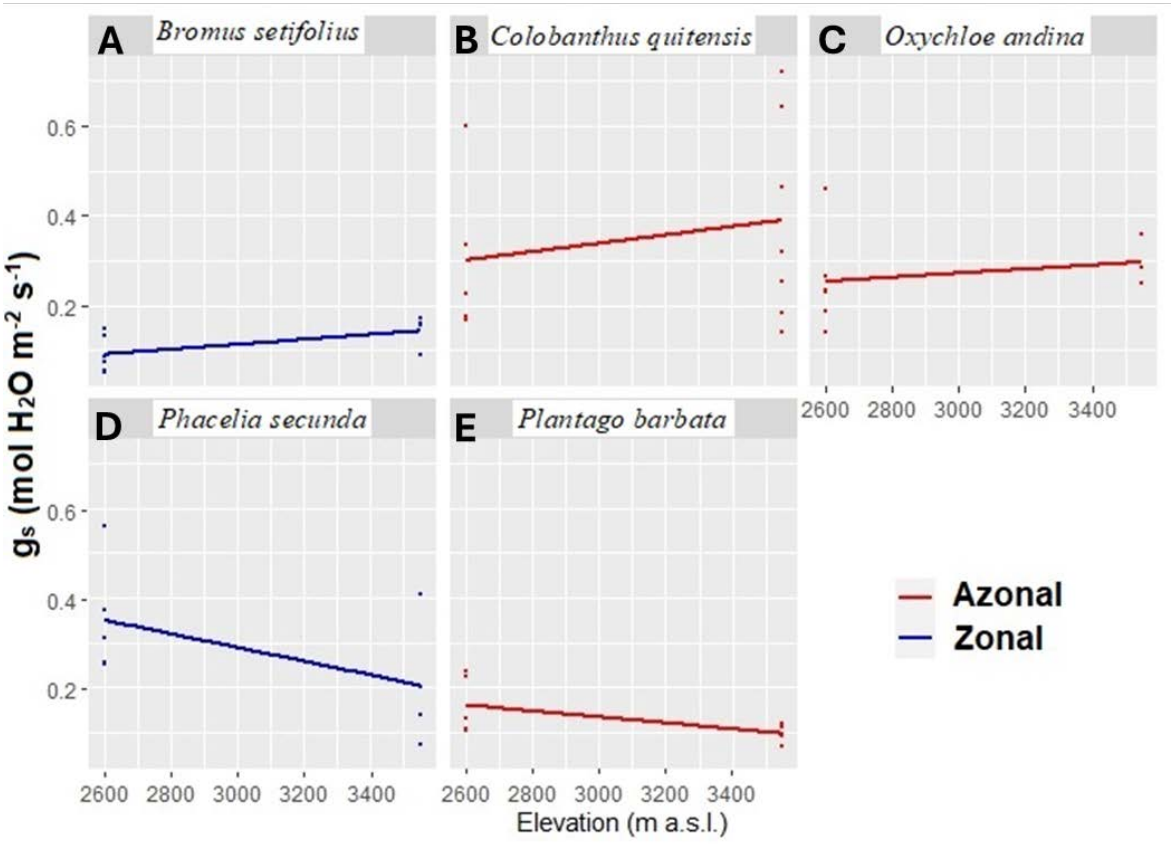

Fig. S 1.3. Dot plot and regression lines of stomatal conductance at different elevations. The graph shows g_s from zonal (blue) *Bromus setifolius* (A) and *Phacelia secunda* (D), and azonal (Red) *Colobanthus quitensis* (B), *Plantago barbata* (C) and *Oxychloe andina* (E) plants from 2600 and 3550 m. a s l.

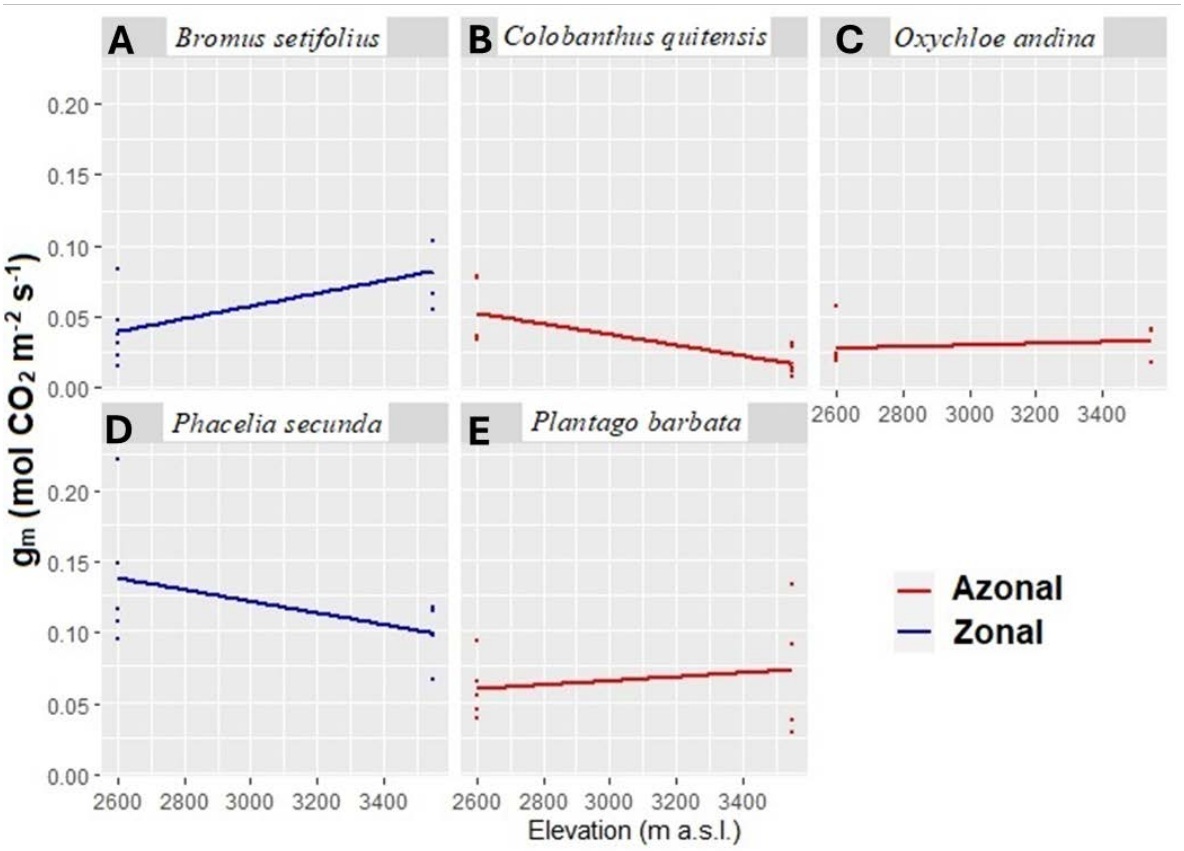

Fig. S 1.4. Dot plot and regression lines of mesophyll conductance at different elevations. The graph shows g_m from zonal (blue) *Bromus setifolius* (A) and *Phacelia secunda* (D), and azonal (Red) *Colobanthus quitensis* (B), *Plantago barbata* (C) and *Oxychloe andina* (E) plants from 2600 and 3550 m. a s l.

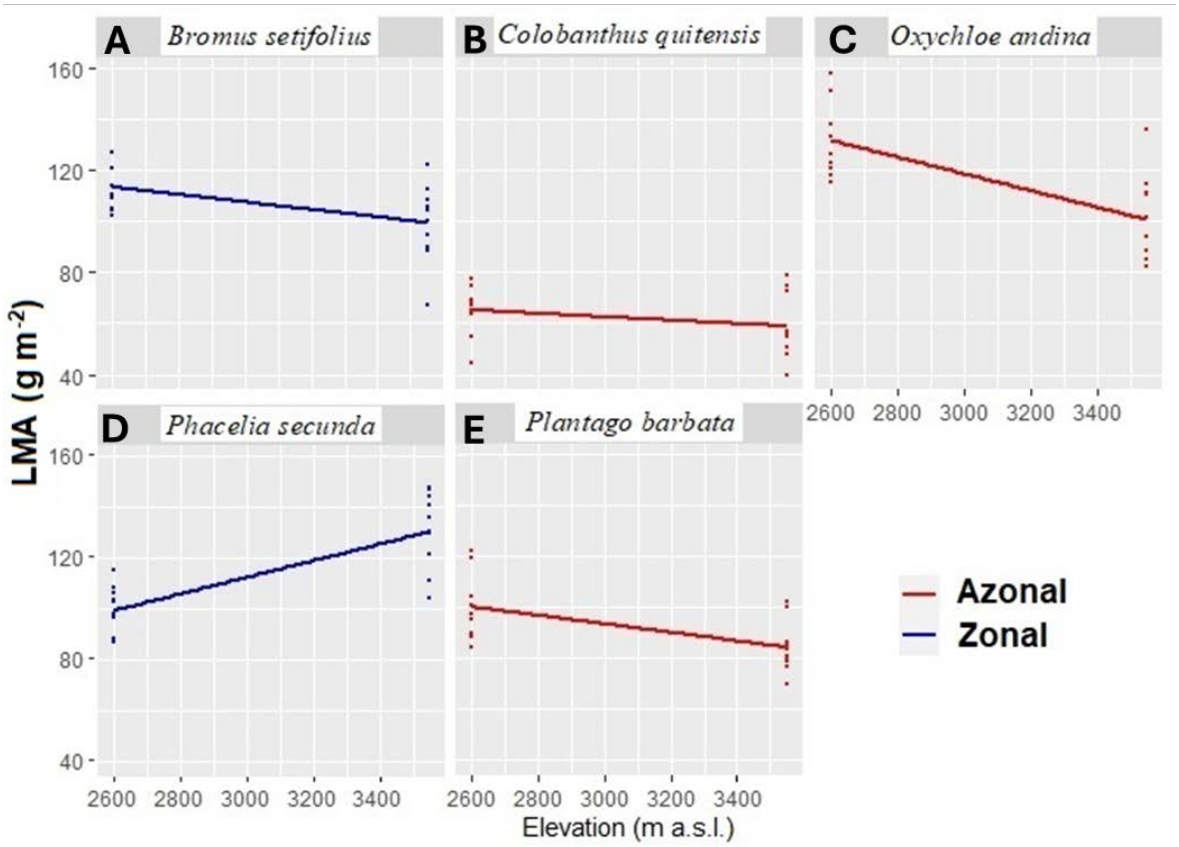

Fig. S 1.5. Dot plot and regression lines of leaf mass per area at different elevations. The graph shows LMA from zonal (blue) *Bromus setifolius* (A) and *Phacelia secunda* (D), and azonal (Red) *Colobanthus quitensis* (B), *Plantago barbata* (C) and *Oxychloe andina* (E) plants from 2600 and 3550 m. a s l.

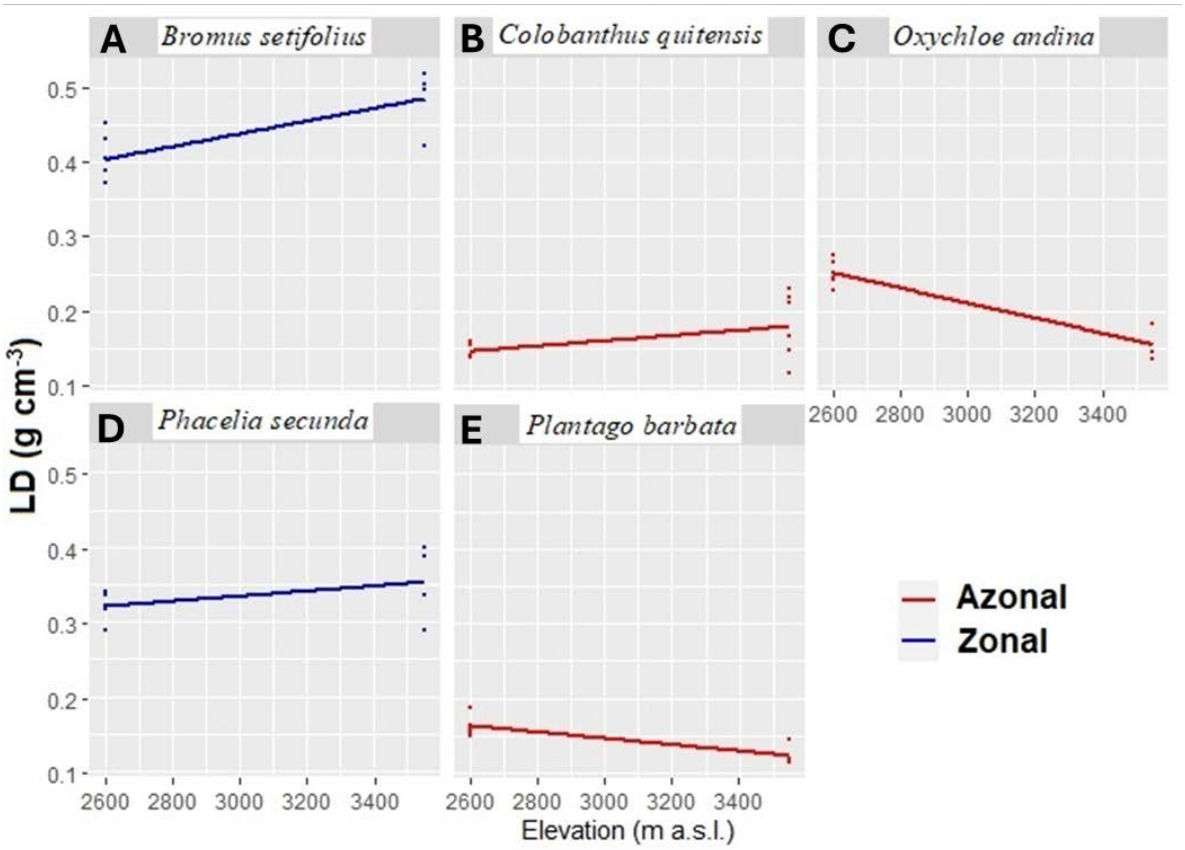

Fig. S 1.6. Dot plot and regression lines of leaf density at different elevations. The graph shows LD from zonal (blue) *Bromus setifolius* (A) and *Phacelia secunda* (D), and azonal (Red) *Colobanthus quitensis* (B), *Plantago barbata* (C) and *Oxychloe andina* (E) plants from 2600 and 3550 m. a s l.

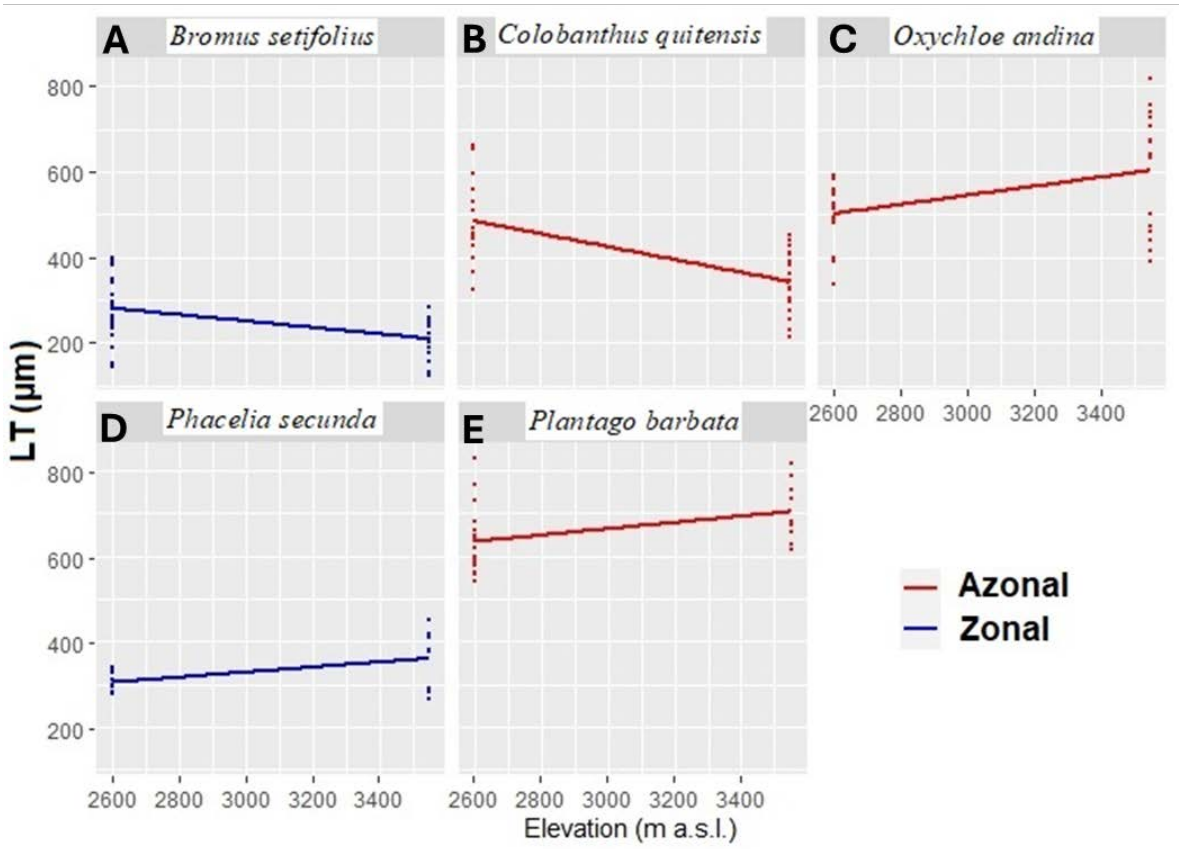

Fig. S 1.7. Dot plot and regression lines of leaf thickness at different elevations. The graph shows LT from zonal (blue) *Bromus setifolius* (A) and *Phacelia secunda* (D), and azonal (Red) *Colobanthus quitensis* (B), *Plantago barbata* (C) and *Oxychloe andina* (E) plants from 2600 and 3550 m. a s l.

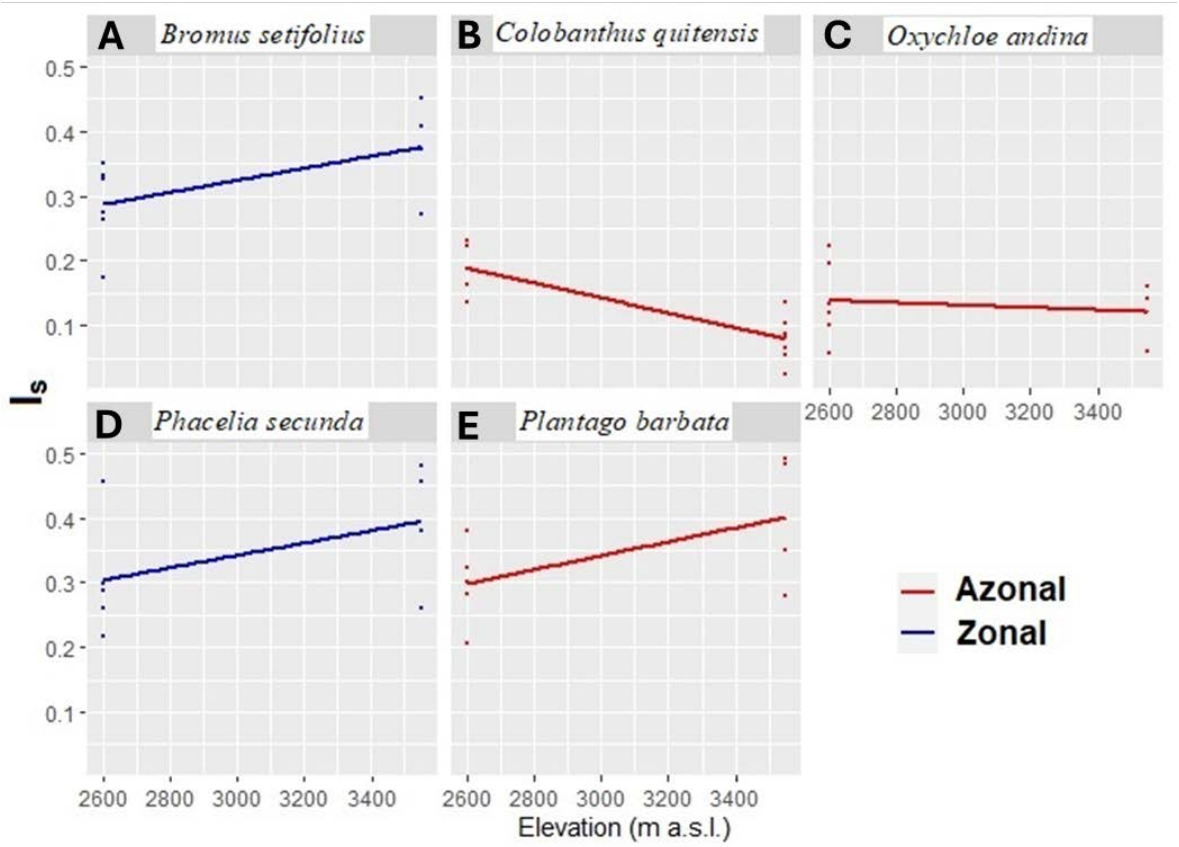

Fig. S 1.8. Dot plot and regression lines of stomatal limitation to photosynthesis at different elevations. The graph shows l_s from zonal (blue) *Bromus setifolius* (A) and *Phacelia secunda* (D), and azonal (Red) *Colobanthus quitensis* (B), *Plantago barbata* (C) and *Oxychloe andina* (E) plants from 2600 and 3550 m. a s l.

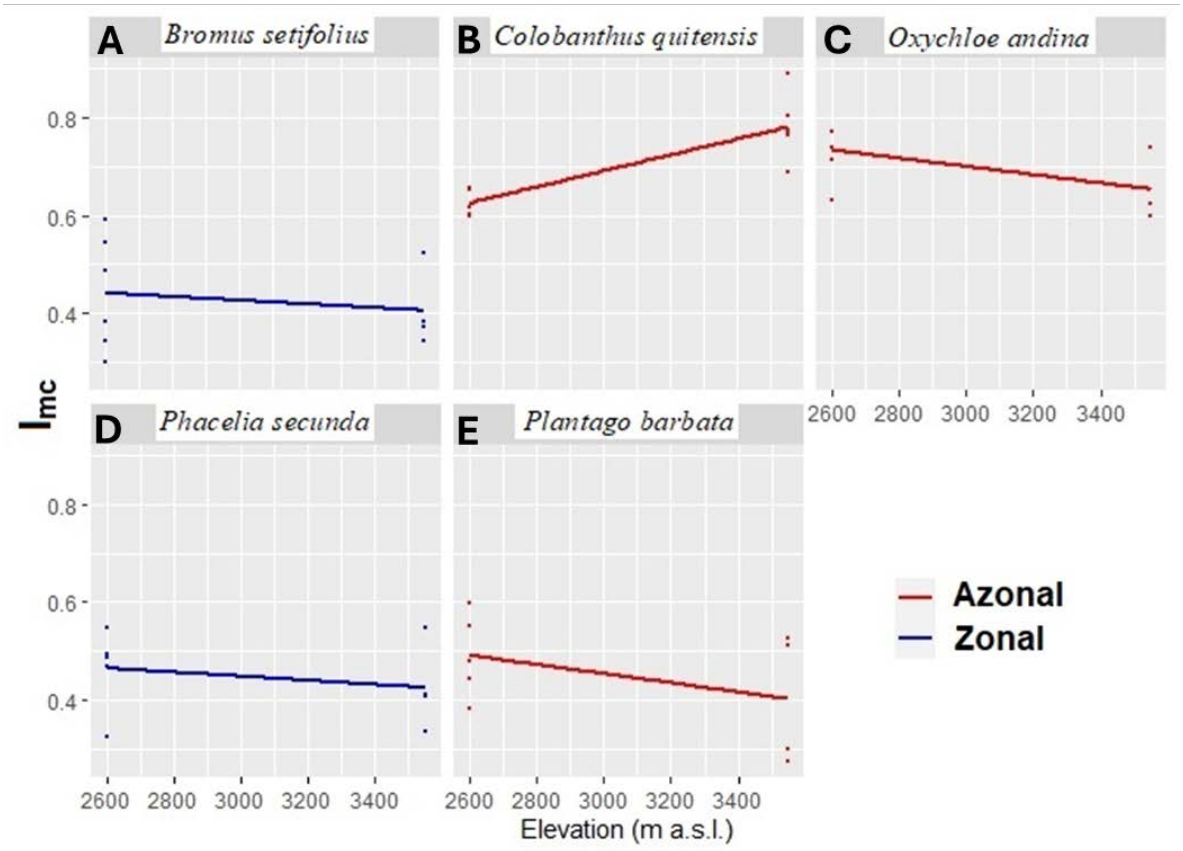

Fig. S 1.9. Dot plot and regression lines of mesophyll limitation to photosynthesis at different elevations. The graph shows l_{mc} from zonal (blue) *Bromus setifolius* (A) and *Phacelia secunda* (D), and azonal (Red) *Colobanthus quitensis* (B), *Plantago barbata* (C) and *Oxychloe andina* (E) plants from 2600 and 3550 m. a s l.

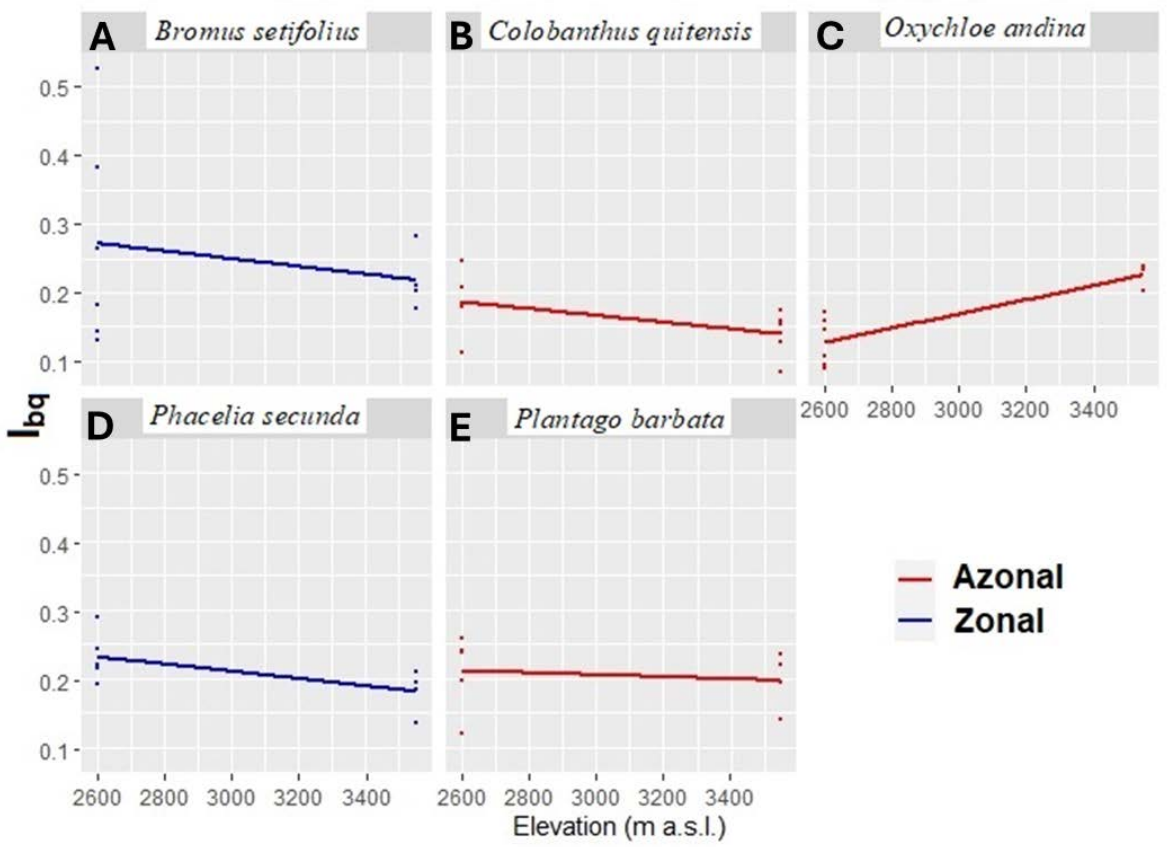

Fig. S 1.10. Dot plot and regression lines of biochemical limitation to photosynthesis at different elevations. The graph shows l_{bq} from zonal (blue) *Bromus setifolius* (A) and *Phacelia secunda* (D), and azonal (Red) *Colobanthus quitensis* (B), *Plantago barbata* (C) and *Oxychloe andina* (E) plants from 2600 and 3550 m. a s l.

Table S 1.2. Anatomical parameters for high-elevation species. Mean mesophyll porosity (Mp), cell wall thickness (Tcw), distance between chloroplast and cell wall (Dccw), chloroplast thickness (Ct), distance between chloroplasts (Dc), surface of exposed mesophyll per area (Sm/S) and surface of exposed chloroplasts per area (Sc/S) for zonal and azonal species from 2600 and 3550 m a.s.l. Statistically significant elevational differences are shown in red ($p<0.05$) according to t or Mann-Whitney test.

Zonal	***Phacelia secunda***		***Bromus setifolius***			
Elevation	**2600**	**3550**	**2600**	**3550**		
Mp (m^3 m^{-3})	0.254	0.266	0.250	0.205		
Tcw (µm)	0.214	0.157	0.379	0.414		
Dccw (m) (*10E-7)	1.090	2.244	1.474	2.242		
Ct (m) (*10E-6)	1.861	2.955	2.172	2.929		
Dc (m) (*10E-7)	2.174	1.163	6.500	1.321		
Sm/s	40.843	47.098	20.884	19.422		
Sc/s	36.913	40.784	16.368	17.062		
Azonal	***Oxychloe andina***		***Plantago barbata***		***Colobanthus quitensis***	
Elevation	**2600**	**3550**	**2600**	**3550**	**2600**	**3550**
Mp (m^3 m^{-3})	0.224	0.189	0.297	0.244	0.227	0.391
Tcw (µm)	0.251	0.320	0.383	0.342	0.293	0.225
Dccw (m) (*10E-7)	1.301	1.438	1.260	1.627	2.001	2.751
Ct (m) (*10E-6)	1.997	2.258	1.389	2.785	2.026	2.335
Dc (m) (*10E-7)	1.501	1.292	6.136	3.287	0.938	8.188

Sm/s	44.303	43.996	47.246	61.932	62.332	42.460
Sc/s	37.730	37.051	36.951	48.958	54.121	27.387

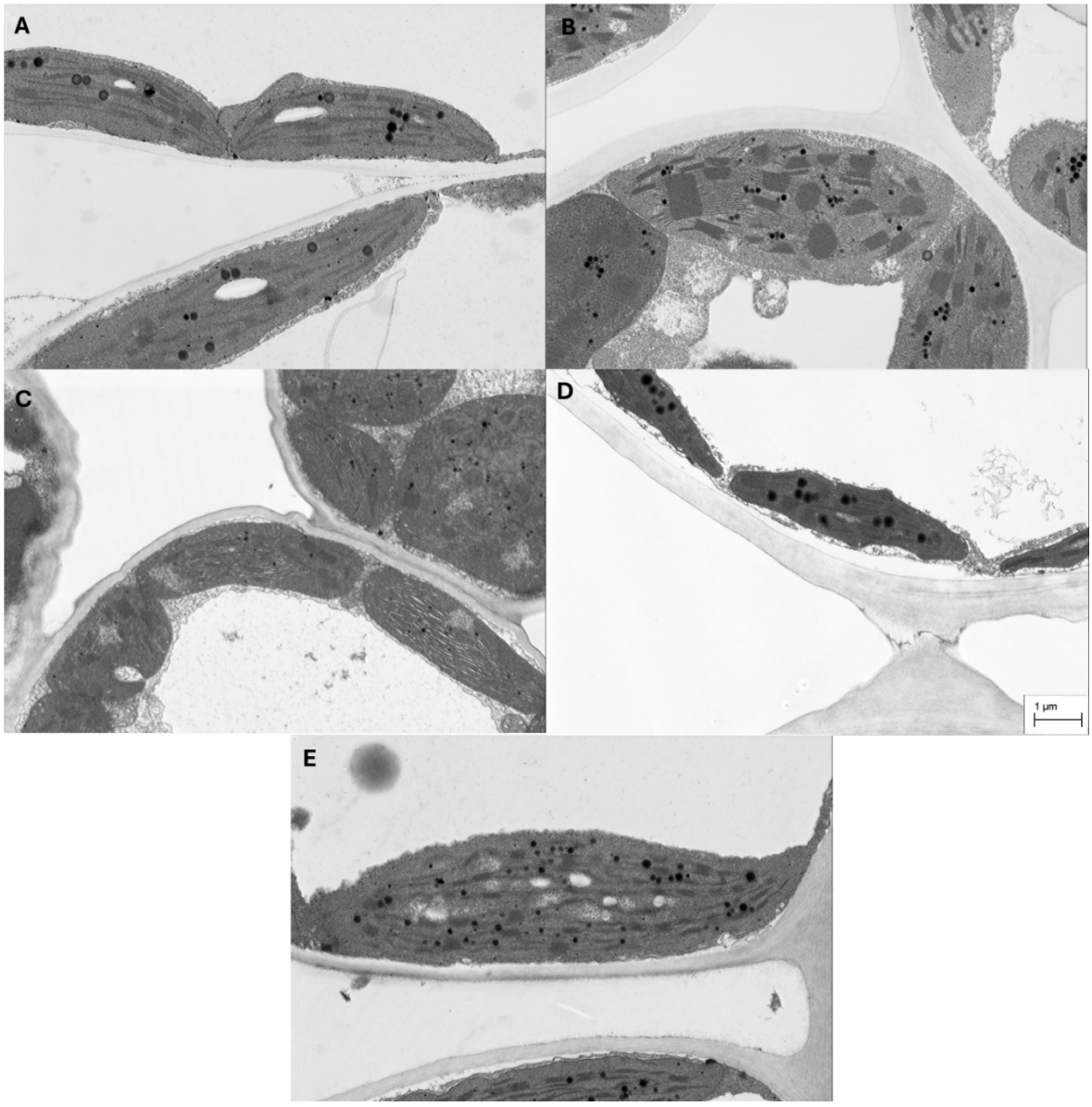

Fig. S 1.11. Micrograph of chloroplasts and cell wall from the 2600 m. a s l species *P. secunda* (A), B. setifolius (B), O. andina (C), P. barbata (D) and C. quitensis (E). All images present the same magnification.

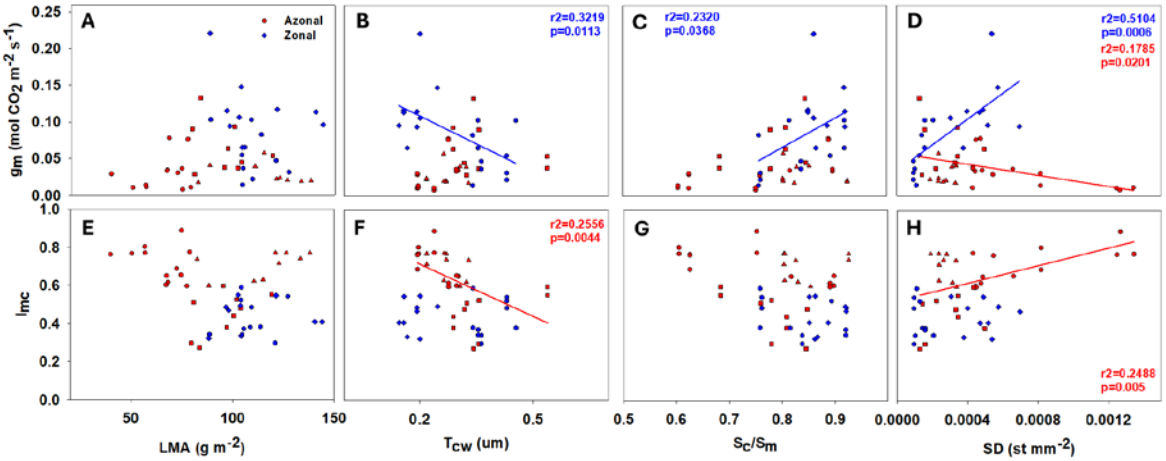

Fig. S 1.12. Regression analysis between mesophyll parameters and anatomical leaf traits in zonal and azonal plants. The graphs show dot plot between leaf mass area (A), cell wall thickness (B), surface of mesophyll occupied by chloroplasts by surface of mesophyll exposed to intercellular air spaces (C) and stomatal density (D) with mesophyll conductance estimated by the Harley method (g_m) and the same parameters with mesophyll limitation (l_{mc}) in panels E, F, G and H respectively. Blue and Red lines represent the regression for zonal and azonal species respectively, coefficient of determination and p values are shown in the graphs.

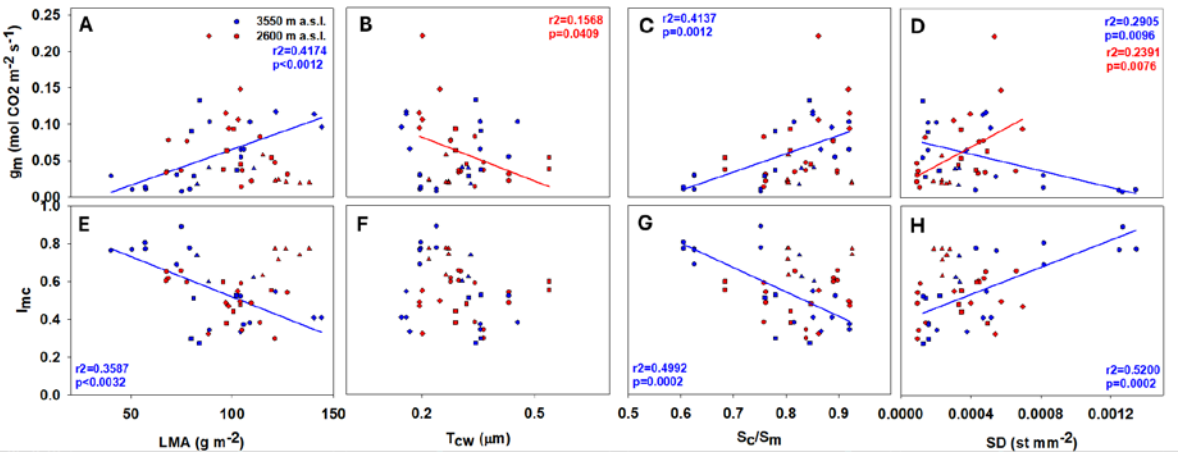

Fig. S 1.13. Regression analysis between mesophyll parameters and anatomical leaf traits for plants from different elevations. The graphs show dot plot between leaf mass area (A), cell wall thickness (B), surface of mesophyll

occupied by chloroplasts by surface of mesophyll exposed to intercellular air spaces (C) and stomatal density (D) with mesophyll conductance estimated by the Harley method (g_m) and the same parameters with mesophyll limitation (l_{mc}) in panels E, F, G and H respectively. Blue lines represent the regressions lines for the high elevation site and red lines represent the regressions for the low elevation site, coefficient of determination and p values are shown in the graphs.

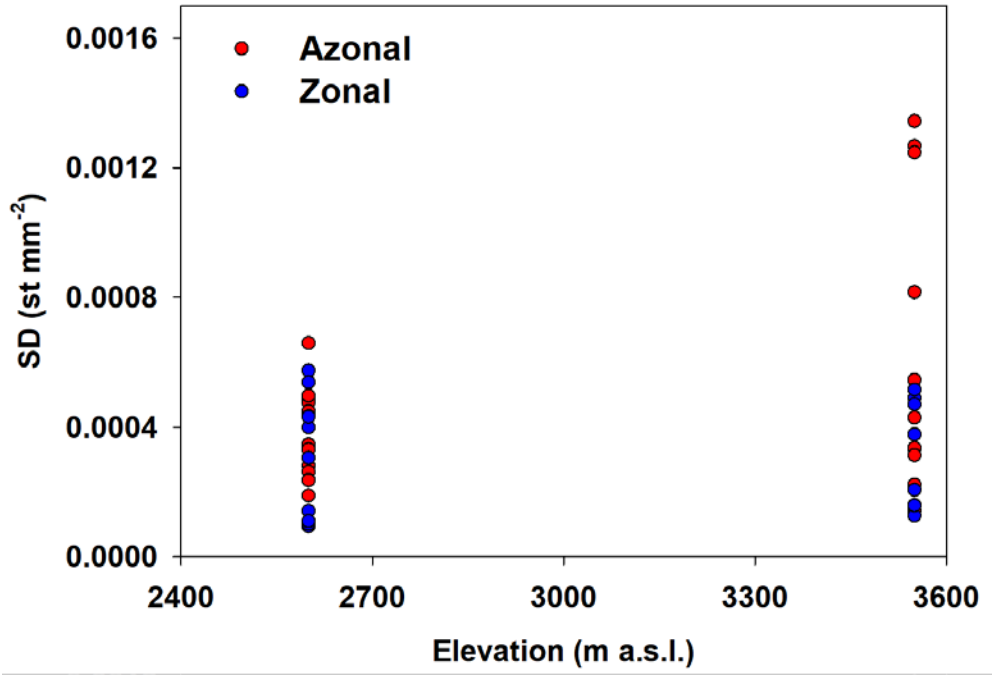

Fig. S 1.14. Dot plot of stomatal density (SD) in two elevations (2600 and 3550 m a.s.l.) from zonal (blue) and azonal (red) plants.

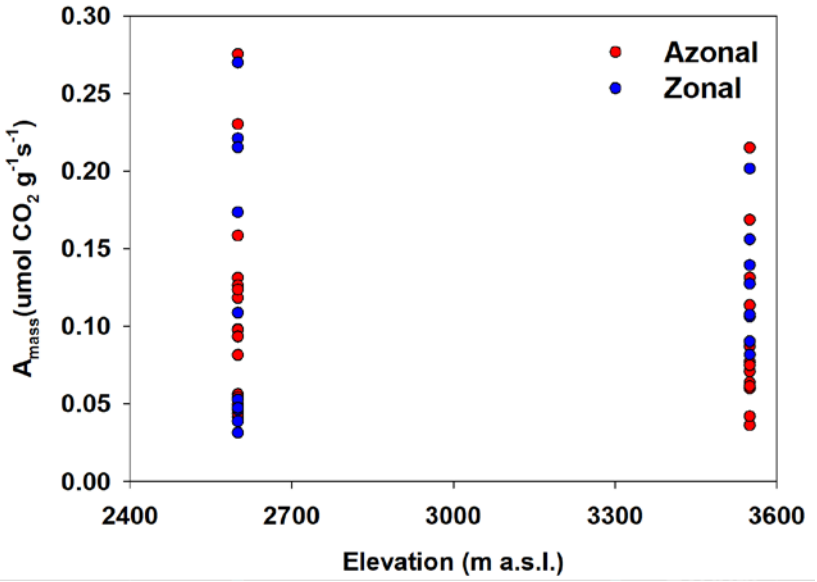

Fig. S 1.15. Dot plot of mass-based photosynthesis (A_{mass}) in two elevations (2600 and 3550 m a.s.l.) from zonal (blue) and azonal (red) plants.

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Capitulo 2

Growth temperature trigger adjustments in net photosynthesis of high-elevation plants from the Andes of Central Chile

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Abstract

Temperature significantly influences plant morphology and photosynthesis, shaping distinct changes with the increase in elevation. Lower temperatures such as those at high elevations typically increase leaf mass per unit area (LMA), potentially reducing mesophyll CO_2 diffusion (gm) and chloroplast CO_2 availability, which can limit photosynthetic rates (A_N). However, many species don't decrease photosynthesis with elevation. Other factors such as CO_2 partial pressure, soil moisture, and light also change with elevation and may be involved in elevational changes in photosynthesis. Habitat type further affects plant responses, as plants inhabiting water saturated substrates (azonal plants) may be more sensitive to thermal changes due to water's buffering effect on temperature fluctuations. To isolate effect of growth temperature on the photosynthetic capacity of high-elevation plants and their determinants, we conducted a controlled experiment with plants collected at two elevations (2600 and 3550 m a.s.l.), exposing them to two daytime temperatures (15 and 20 °C). We measured LMA, g_m, and A_N. We expected that plants from low-elevation decreased their photosynthesis when grown at low temperature (15°C), whilst plants from high-elevation increased their photosynthesis when grown at high-temperature (20°C). Growth temperature significantly influenced photosynthesis and g_m but not LMA. Elevation provenance and habitat type (zonal vs. azonal) also shaped responses. Our findings reveal that temperature impacts photosynthesis by altering mesophyll diffusion independently of LMA. Furthermore, adaptations to specific elevations and environments, such as wetlands, contribute to the variability in responses. This study highlights the nuanced role of temperature in shaping photosynthetic traits in high-elevation plants.

Keywords: alpine plants, elevation, LMA, mesophyll conductance, photosynthesis**,** temperature, wetland**.**

Introduction

Decreased air temperature is one of the primary changes associated with increase in elevation (Körner, 2021). It has been suggested that this elevational decrease in temperature affects key physiological processes such as photosynthesis (Gratani et al., 2014; Kao & Chang, 2001; Kogami et al., 2001; Ma et al., 2015; Shi et al., 2015; Viveros et al., 2024). For instance, Shi et al. (2015) associated the lower leaf temperatures recorded at high elevation with lower photosynthetic rates due to low g_m in evergreen trees. Similarly, reduced photosynthetic rates at high elevations were attributed to lower temperatures in *Miscanthus* species, associated with higher LMA, proposing an influence on g_m (Kao and Chang 2001). Lower A_N in *Sesleria nitida* was also associated to lower temperatures at higher elevation in Gratani et al. (2014). Conversely, working with shrubs and grasses, Shi et al. (2015) reported higher photosynthetic rates at higher elevations, associated with higher biochemical capacity and total conductance (g_s and g_m). The increased A_N and biochemical capacity with elevation has been found in other studies as well (Fan et al., 2011; Kostopaulou and Karatassiou 2016). Nonetheless, there are other works reporting that photosynthetic rates along elevational gradients are higher at intermediate elevations due to biochemical and anatomical adaptations, but A_N finally decreases at the higher elevation due to low temperatures and a higher investment in survivability proteins instead of photosystems (Ma et al. 2015, Xing et al. 2024). Thus, there is no general pattern regarding changes in photosynthesis with temperature along elevations. This is likely because, with elevation, other factors such as water availability, irradiance and partial pressure of gases also change, imposing diverse effects on the photosynthetic process (Evans & Poorter, 2001; Flexas et al., 2004; Gago et al., 2020; Tosens et al., 2011). Thus, although the temperature appears to be a significant determinant of photosynthesis in high-elevation plants under field conditions, it is difficult to isolate its effects from other environmental factors that also change with elevation.

Temperature is thought to induce changes in foliar structure. For instance, Dong et al. (2020) showed increases in leaf mass area (LMA) with lower temperatures

across over 700 species in Australia, similar to the results for *Quercus ilex* across 195 sites along Spain (Ogaya and Peñuelas 2007). The meta-analysis of Poorter et al. (2009) also showed that LMA tends to increase with decreasing temperatures. In the mountains of China, Zhang et al. (2020) observed increases in LMA along a gradient of around 450 m, which was attributed to decrease in temperature, given that factors such as light and relative soil moisture were similar across different elevations. Increases in LMA could affect photosynthesis in two ways: enhancing photosynthesis due to higher leaf nitrogen per area, which could positively influence key photosynthetic parameters such as maximum carboxylation rate (V_{cmax}) and electron transport rate (J_{max}) (Shi et al., 2015). Conversely, higher LMA could reduce photosynthesis because a higher LMA is often associated with lower g_m (Onoda et al., 2017), as a thicker leaf increases the diffusion distance of CO_2 to the chloroplasts. Additionally, higher LMA could be influenced by increased in cell wall thickness, which may limit the CO_2 diffusion in the liquid phase (Tomás et al., 2013).

Recent studies have highlighted the significant role of g_m as a limiting factor for photosynthesis in mountain plants (e.g. Viveros et al., 2024; Xing et al., 2024). The analysis of photosynthetic limitations evaluates the balance between g_s, g_m and the biochemical capacity of the leaf, determining the relative importance of each limitation, which has been observed to vary in response to different environmental conditions such as temperature, water, or nutrient availability (Niinemets et al., 2009; Gago et al., 2020). Therefore, decreases in temperature as those found along elevational gradients, could reduce photosynthesis in mountain plants through increased LMA, triggering a greater mesophyll limitation.

In mountains located in mediterranean-type regions, high-elevation plants are exposed to drought, especially at the end of the growing season. However, there are azonal vegetation formations where plants do not experience drought throughout the growing season, as they grow in water saturated soils that receive water from glaciers, melt runoff or groundwater outcrops. This contrasts with plants in zonal formations that grow in areas exposed to seasonal droughts. In a previous study, we reported that the LMA and photosynthetic characteristics such as A_N and g_m of zonal

and azonal species responded differently to elevation (Viveros et al., 2024). Also, when comparing wetland and non-wetland plant species, Pan et al. (2020) found differences in LMA, being lower in wetland species. Further, according to the leaf economic spectrum, lower LMA could be related to vulnerability to harsh environmental conditions (Onoda et al., 2017) such as low temperatures. Additionally, azonal plants, growing in water-saturated environments, may experience greater thermal stability due to the high specific heat of water and thus could be more sensitive to changes in leaf temperature, as showed by Ruthzatz (1978), where surface soil from zonal species could reach 40°C in high Andean environments, while in wetlands the temperature never surpassed 25°C.

In the Andes of Central Chile, plants can grow up to 3600 m a.s.l. At this elevation, daytime air temperatures during growing season are around 15°C, while one kilometer downward in elevation temperatures are around 20°C (Cavieres et al., 2007). In different mountains, this altitudinal difference had been shown to be sufficient to induce changes in various foliar traits, such as LMA and photosynthetic rates (Ma et al., 2015; Shi et al., 2015; Viveros et al., 2024). However, there is no clear understanding of the factors underpinning these changes. We hypothesized that temperature drives the lower photosynthetic rates in high-elevation plants, and the mechanism underlying temperature associated variations in A_N involves changes in LMA and consequently in g_m. Thus, when plants from high elevation are grown at higher temperatures, we hypothesize that they reduce their LMA, resulting in higher g_m, lower mesophyll limitation, and therefore higher A_N. Conversely, plants from lower elevations, when grown at lower temperature, increase their LMA, leading to decreased g_m, higher mesophyll limitation, and hence lower A_N. Considering the high thermal stability and lower LMA of plants growing in azonal vegetation, we also hypothesized that the effect of changes in growth temperature are bigger in azonal plants compared to zonal plants.

Material and methods

Experimental design

Zonal and azonal plants species growing at two elevations (2600 and 3550 m a.s.l.) in the Central Chile Andes (33.19'40''S, 70.17'33'' W and 33.19'34'' S, 70.15'15'' W) were collected and transferred to the laboratory and cultivated in a greenhouse, in pots of 500 ml, in a substrate consisting of perlite, peat, and sand in a 1:3:3 v/v. Zonal species corresponded to *Phacelia secunda* and *Bromus setifolius*, whilst azonal plants species were *Plantago barbata*, *Oxychloe andina*, and *Colobanthus quitensis*. Plants were watered periodically and fertilized every three weeks. After 6 weeks, zonal and azonal plants from both elevations were randomly assigned to two growth temperature regimes: 15 °C and 20 °C, with similar night-time temperature of 10 °C. These temperatures were chosen because they corresponded to the mean midday air temperatures at 10 cm aboveground during the growing season at 2600 and 3550 m a.s.l., respectively (Cavieres et al. 2007). Plants were kept at each temperature regime for at least 3 months before measurements to allow them to fully acclimate and develop new leaves in the set growth conditions.

Chlorophyll fluorescence and gas exchange

Leaf gas exchange was measured simultaneously with chlorophyll fluorescence using a LI-6400XT gas exchange system (LI-COR Inc., Lincoln, NE, USA) with an integrated fluorescence chamber (LI-6400-40; LI-COR Inc.). Measurements were taken on a single leaf or a group of leaves, aiming to cover the entire chamber area without overlap, following the procedure described by Sáez et al. (2017). Leaf area was determined using ImageJ software (Wayne Rasband/NIH, Bethesda, MD, USA) by taking a photograph and measuring the area within the chamber. Conditions within the chamber included photosynthetically active radiation (PAR) of 1500 µmol photons m^{-2} s^{-1} for all species, except *C. quitensis*, which was set at 1000 µmol photons m^{-2} s^{-1}, with 10% blue light, relative humidity between 40 and 60%, and leaf temperature fixed at 15°C and 20°C according to growth temperature. Point corrections for CO_2 leakage were applied to all gas exchange measurements according to Flexas et al. (2007). The quantum efficiency of photosystem II (PSII) was determined according to the equation:

$$\phi PSII = \frac{(F'm - F's)}{F'm}$$

where F's represents the steady-state fluorescence under constant light (1500 μmol m^{-2} s^{-1}), and F'm represents the maximum fluorescence obtained after a saturating light pulse of 8000 μmol m^{-2} s^{-1} for 0.8 seconds. Since ϕPSII represents the number of electrons transferred per photon absorbed in PSII, the electron transport rate (ETR) can be calculated as ETR = φPSII · PPFD · αβ, where PPFD is the photosynthetic photon flux density, and αβ is a standardized parameter set at 0.45.

Mesophyll conductance estimation

The mesophyll conductance to CO_2 was calculated based on the combination of gas exchange and chlorophyll fluorescence, following the method described by Harley et al. (1992):

$$g_m = \frac{A_N}{C_i - \frac{(\Gamma(ETR + 8(A_N + R_n))}{(ETR - 4(A_N + R_l)))}}$$

A_N and C_i were obtained from gas exchange measurements under saturating light. The rate of non-photorespiratory CO_2 evolution under light (Rl) was assumed to be half of the dark respiration (Rn). The CO_2 compensation point (Γ) was calculated taking into account the temperature effect over Γ according to Bernacci et al. (2002) and the altitudinal reduction of CO_2 partial pressure (Farquhar et al., 1980). The determination of gm was used to calculate Cc.

Leaf mass per area ratio

At each growth temperature, provenance, and species, 10 leaves were collected and photographed. Their area was measured using ImageJ software (Wayne Rasband/NIH, Bethesda, MD, USA). The leaves were then dried in an oven at 70°C

for 48 hours to measure their dry mass and calculate LMA by dividing leaf mass by area.

Quantitative analysis of photosynthetic limitations

To separate the relative control of A_N from the limitations imposed by stomatal conductance (gs), mesophyll conductance (gm), and biochemical capacity (lb) (gs + gm + lb = 1), the quantitative analysis of Jones et al. (1985) was used, as implemented by Grassi and Magnani (2005). The limitation of the different components was calculated as:

$$l_s = \frac{(g_{tot}/g_s)\delta A_N/\delta C_c}{g_{tot} + \delta A_N/\delta C_c},$$

$$l_{mc} = \frac{(g_{tot}/g_m)\delta A_N/\delta C_c}{g_{tot} + \delta A_N/\delta C_c},$$

$$l_{bq} = \frac{g_{tot}}{g_{tot} + \delta A_N/\delta C_c},$$

where gs is the stomatal conductance, gm is the mesophyll conductance according to Harley et al. (1992), and gtot is the total conductance for CO_2 from the ambient air to the chloroplasts, which was calculated using the following equation:

$$g_{tot} = \frac{1}{(1/g_s + 1/g_m)}$$

Statistical analysis

Linear mixed-effects models were used to evaluate the effects of plant provenance (2600 and 3550 m a.s.l.) and growth temperature (15°C and 20°C) on LMA, g_m, A_N, g_s, and photosynthetic limitations. In these models, species identity was included as a random factor to account for the interdependence of observations from the same

species. The stomatal conductance (gs), and the stomatal and biochemical limitations (ls and lb) were log-transformed to reduce deviation from normality. Individual observations are presented untransformed to facilitate the visualization of results. Probability values were obtained from Wald's Type II chi-square test. The normality of residuals was visually assessed using quantile-quantile plots and residual distribution plots. A Tukey post-hoc test was applied to identify specific differences between groups where there was an effect of both fixed factors and their interaction. Mixed models were conducted using the lme4 package in R statistical software (v. 4.4.1; R Core Team).

Results

Photosynthetic leaf gas exchange

Mean photosynthetic rates ranged from 3.3 to 7.5 μmol CO_2 m^{-2} s^{-1} and were negatively affected by growth temperature in both zonal and azonal plants, regardless elevational provenance (Fig. 2.1A-B). Interestingly, g_m responded differently to growth temperature in zonal and azonal plants: zonal plants did not show effects of growth temperature, whereas azonal plants exhibited lower gm values at higher growth temperatures (Fig. 2.1C-D). The elevational provenance effect was not significant in most variables except for g_s. Mean stomatal conductance ranged from 0.08 to 0.19 mol H_2O m^{-2} s^{-1}. In azonal plants g_s was only negatively affected by higher growth temperature, regardless elevation provenance, while zonal plants showed a provenance effect, with plants from higher elevation showing higher g_s (Fig. 2.1E-F). Mean LMA ranged from 82 to 117 g m^{-2}, being higher in zonal plants compared to azonal plants. However, within these groups, LMA was not significantly affected by growth temperature or plant provenance (Fig. 2.2A-B; Table 2.1).

Table 2.1 Effects of plant provenance (2600 and 3550 m a.s.l.) and growth temperature (15-20°C) on leaf structure and photosynthetic leaf gas exchange

of zonal and azonal plants from the Central Andes of Chile. Significant p-values (< 0.05) from the linear mixed-effects models are presented in bold.

Variable	Factor	Chi Sqr	p-value
LMA zonal	Provenance	1.138	0.286
	Temperature	0.131	0.716
	Provenance:Temperature	0.164	0.685
LMA azonal	Provenance	0.993	0.318
	Temperature	0.050	0.822
	Provenance:Temperature	0.107	0.743
g_m zonal	Provenance	0.674	0.411
	Temperature	0.372	0.541
	Provenance:Temperature	1.266	0.260
g_m azonal	Provenance	0.388	0.532
	Temperature	4.316	**0.037**
	Provenance:Temperature	2.097	0.147
A_N zonal	Provenance	0.431	0.431
	Temperature	7.872	**0.005**
	Provenance:Temperature	0.089	0.764
A_N azonal	Provenance	2.621	0.105
	Temperature	23.08	**0.000**
	Provenance:Temperature	0.294	0.587
g_s zonal	Provenance	5.584	**0.018**
	Temperature	0.082	0.774
	Provenance:Temperature	0.578	0.446
g_s azonal	Provenance	0.021	0.883
	Temperature	31.88	**0.000**
	Provenance:Temperature	0.310	0.577

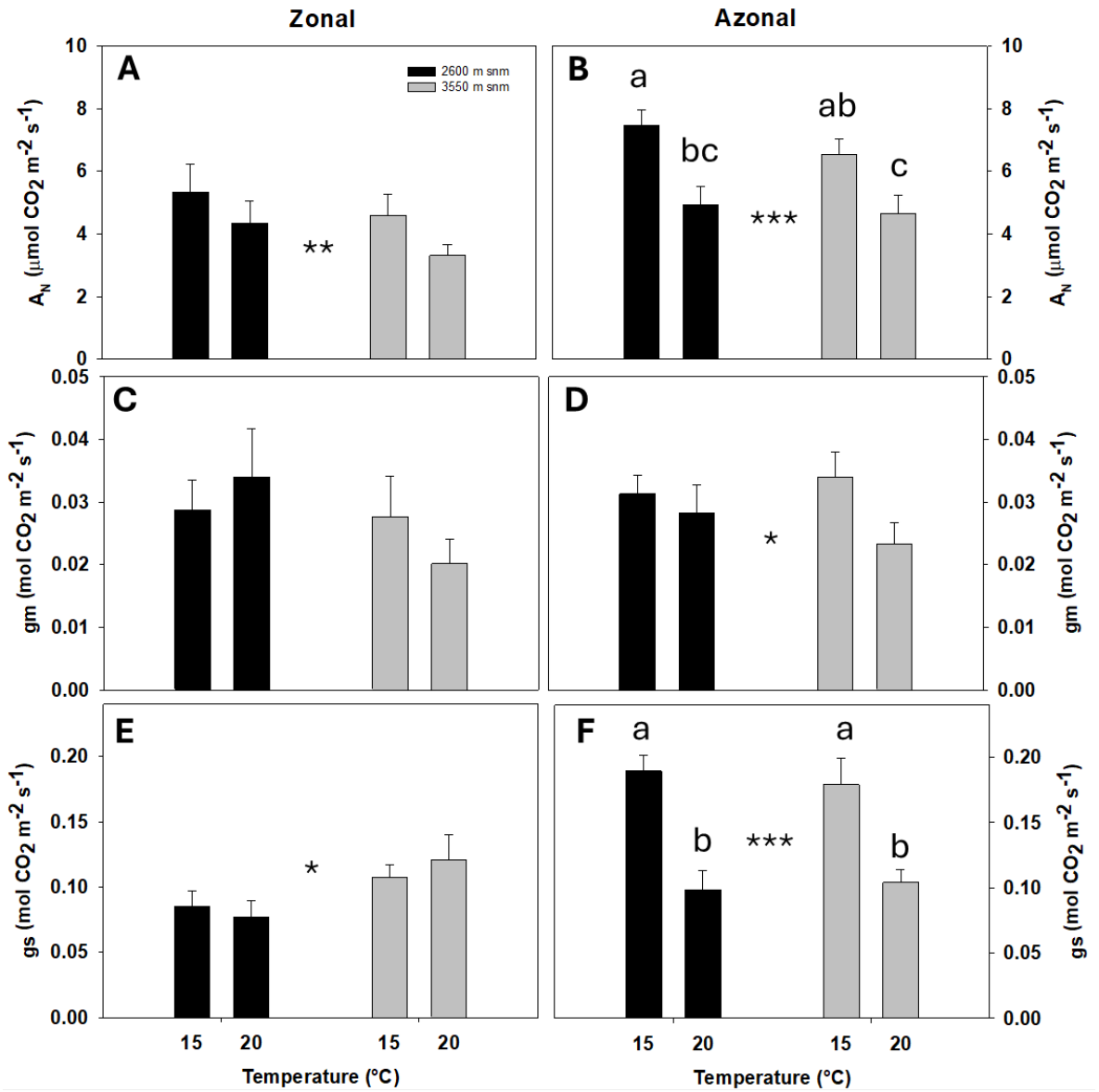

Fig. 2.1. Photosynthetic response to growth temperature. Response of mean A_N (A-B), g_m (C-D) and g_s (E-F) to two growth temperatures (15 - 20°C) in zonal (left) and azonal (right) plants from two contrasting elevations, 2600 (black) and 3550 m a.s.l. (gray) n=6 for each bar, SE is presented. Asterisks indicate significant effects of temperature or provenance ($p < 0.05$) according to the Wald test for the linear mixed-effects models.

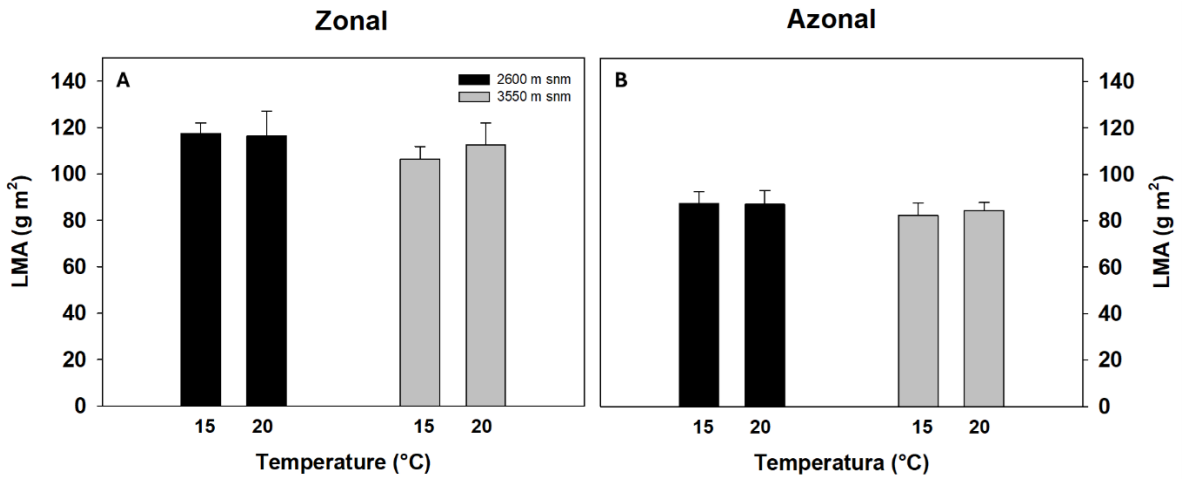

Fig. 2.2. LMA response to growth temperature. Response of LMA (A-B) to two growth temperatures (15 - 20°C) in zonal (left) and azonal (right) plants from two contrasting elevations, 2600 (black) and 3550 m a.s.l. (gray). n=8 for each bar, SE is presented. Statistical differences were evaluated through Wald test for the linear mixed-effects models.

Photosynthetic limitations

In zonal plants, stomatal limitation (ls) was lower in plants from higher elevations and showed a decrease when grown at 20°C (Table 2.2; Fig. 2.2A). Conversely, temperature affected the ls of azonal plants differently based on provenance, increasing with temperature in plants from 2600 m a.s.l., while no temperature effect was observed in plants from 3550 m a.s.l. (Fig. 2.2B). Mesophyll conductance limitation (lm) was not affected by temperature or provenance in zonal plants. In azonal plants, however, plants from different provenances responded differently to temperature; while plants from 2600 m a.s.l. showed decreased gm at higher temperature, plants from 3550 m a.s.l. did not exhibit changes in gm (Fig. 2.2C-D). Finally, the biochemical limitation (lb) in zonal plants increased at higher temperature. Conversely, lb in azonal plants varied with provenance, increasing in plants from 2600 m a.s.l. but remaining unchanged in plants from 3550 m a.s.l. (Fig. 2.2E-F).

Table 2.2 Effects of plant provenance (2600 and 3550 m a.s.l.) and growth temperature (15-20°C) on the photosynthetic limitations of zonal and azonal plants from the Central Andes of Chile. Significant p-values (< 0.05) from the linear mixed-effects models are presented in bold.

Variable	Factor	Chi Sqr	p-value
ls zonal	Provenance	5.943	**0.0147**
	Temperature	4.812	**0.0282**
	Provenance:Temperature	0.105	0.7458
ls azonal	Provenance	0.351	0.5531
	Temperature	2.706	0.0999
	Provenance:Temperature	4.566	**0.0326**
lm zonal	Provenance	1.453	0.2280
	Temperature	0.929	0.3350
	Provenance:Temperature	0.025	0.8733
lm azonal	Provenance	0.011	0.9135
	Temperature	6.399	**0.0114**
	Provenance:Temperature	8.862	**0.0029**
lb zonal	Provenance	0.015	0.9008
	Temperature	8.062	**0.0045**
	Provenance:Temperature	0.200	0.6545
lb azonal	Provenance	0.841	0.3589
	Temperature	3.604	0.0576
	Provenance:Temperature	3.940	**0.0471**

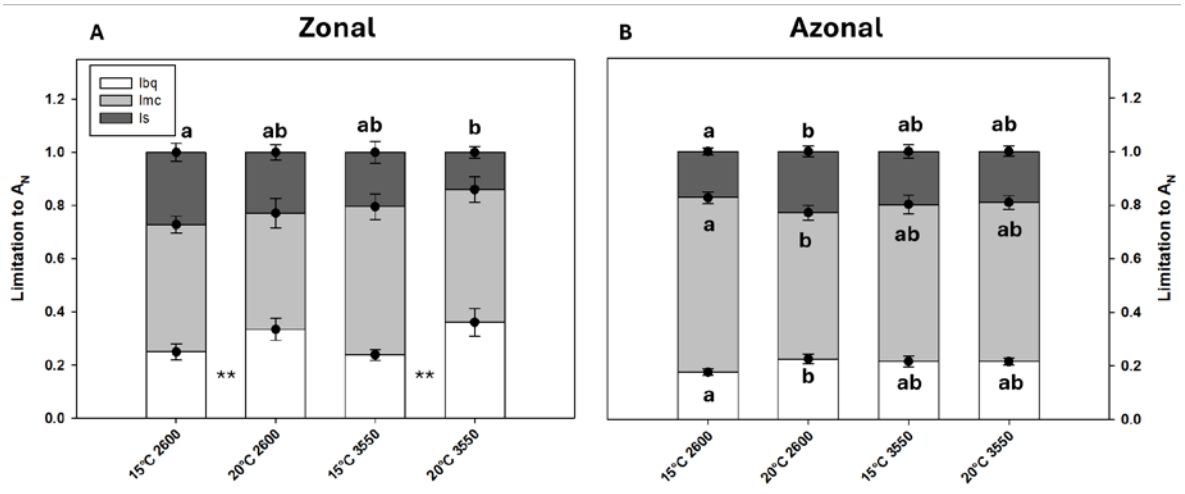

Fig. 2.3. Photosynthetic limitations response to growth temperature. Mean Photosynthetic limitations: ls (A-B), lm (C-D), and lb (E-F) at two growth temperatures (15 - 20°C) in zonal (left) and azonal (right) plants from two contrasting

elevations, 2600 (black) and 3550 m a.s.l. (gray). n=6 for each bar, SE is presented. Asterisks indicate significant effects of temperature or provenance ($p < 0.05$) according to the Wald test for the linear mixed-effects models. In cases where there is an effect of both fixed factors or their interaction, the plots show differences between means from the Tukey test ($p > 0.05$) in different letters when comparing the same limitation in different treatments.

Discussion

We hypothesized that in plants from two contrasting elevations, changes in growth temperature would lead to variation in LMA, resulting in greater limitation by mesophyll conductance (gm) and lower photosynthesis rates in plants from low elevation when grown at low temperature and the opposite for plants from higher elevation grown at high temperature. This effect was expected to be more pronounced in azonal plants due to the greater thermal stability of their environment. According to our results, the hypothesis was rejected, as LMA did not change with different growth temperatures in both elevation provenances. This contrast to observations from meta-analysis studies (Poorter et al., 2009) where LMA change with temperature. Nonetheless, LMA changes in field studies with alpine plant species has been attributed to other factors such as light availability, decreased leaf expansion or CO_2 partial pressure (Shi et al., 2015; Viveros et al., 2024; Kao and Chang 2001). Thus, the lack of LMA changes with the growing temperature observed in our study suggest that factors similar to those mentioned above could be involved in the determination of LMA in high-elevation species of the central Chile Andes. Further, the time of the growing season could drastically affect LMA values in the field, as LMA can double its value depending on the time of the sample collection (Viveros et al., unpublished).

Despite the growth temperature did not change LMA, it exerted different effects on both g_m and photosynthesis in the high-elevation species analyzed here. These results are in line with recent studies (e.g. Rahimi-Majd et al., 2024) where it has been shown that, in C_3 grasses, the best predictors of g_m are factors related to

chloroplast arrangement and cell wall thickness, rather than LMA. Field studies in altitudinal gradients from the Andes also show that g_m correlates mainly with chloroplast arrangement and cell wall thickness (Viveros et al., 2024).

Interestingly, higher growth temperatures in plants from high elevation negatively affected the photosynthesis of the studied species. This could be due to the adaptation of the biochemical apparatus of high elevation plants to lower growth temperatures. This agrees with the work from Bunce et al. (2000) where cold-climate plants grown at lower temperatures deployed higher Vcmax and Jmax, and Xing et al. (2024) who found lower biochemical limitations at higher elevations. It is likely that at higher elevations plants may have enhanced biochemical capacities due to higher nitrogen content per area and greater RuBisCO carboxylation rate (Vcmax) and electron transport rate (Jmax) (Korner & Diemer, 1987; Peng et al., 2020).

On the other hand, plants from low elevation also showed high A_N when grown at lower temperature, even when they naturally grow at higher temperatures during the growing season. This could be explained by the fact that plants present the highest photosynthetic rate at the beginning of the growing season and these values decrease as the temperature rises in the Chilean central Andes (Reyes-Bahamonde et al., 2022). Also, Wrinkler et al. (2018) found higher A_N at the beginning of the growing season at 3545 m a.s.l. in the Rocky Mountains right after snowmelt. Therefore, high-elevation plants may have a wide growth-temperature relationship being adapted to low growth temperatures, such as those present at the beginning of the growing season and thus decrease A_N when grown at higher temperatures.

As expected, zonal and azonal plants responded differently to growth temperature. While zonal plants did not show significant effects of temperature on g_m or stomatal conductance (g_s), azonal plants adjusted both, accompanied by decreasing A_N. This is in line with the study of Pan et al. (2020), where plants from non-wetland areas, such as our zonal species, had higher LMA compared to wetlands plants as our azonal plants. These higher LMA were consistently associated with lower A_N (5.8 μmol CO_2 m^{-2} s^{-1} in azonal plants vs. 4.3 μmol CO_2 m^{-}

^{2} s^{-1} in zonal plants). Additionally, with higher A_N at low temperature, azonal plants experienced greater reductions in A_N when this increased (32% decrease in azonal plants vs. 23% decrease in zonal plants), which aligns with a greater sensitivity of these species to temperature changes. It is worth noting that the water in the azonal formations originates from snow melting runoff and underwater outcrops from snow and glacier melting and therefore is at low temperature. Thus, this may explain that zonal and azonal plants have different responses to temperature, with azonal plants being more sensitive to increases in growth temperature.

Another interesting finding was the effect of provenance on g_s in zonal plants. Plants from 3550 m a.s.l. showed higher gs values compared to plants from 2600 m a.s.l. This effect has been observed in other studies (Gurevitch, 1992; Ma et al., 2015; Shi et al., 2015, Hernandez-Fuentes et al., 2023), being proposed that higher g_s and stomatal density could act as a compensatory mechanism to cope with the lower CO_2 partial pressure at higher elevations (Kouwenberg et al., 2007; Woodward et al., 2002). On the other hand, higher g_s could naturally result from smaller cell sizes and reduced leaf expansion, as the stomata would be concentrated in smaller leaf areas, leading to higher diffusion rates per area. In any case, our results show that, regardless of temperature, zonal plants from higher elevation have higher g_s values, whereas this is not the case for azonal plants, likely because these plants don't lack water supply and hence tend to have high g_s values across different elevations.

Regarding the factors limiting photosynthesis at different growth temperatures, the stomatal limitation (ls) in zonal plants was lower at higher temperature and exhibited a provenance effect, being lower in plants from higher elevation, consistent with the observed g_s. In these zonal plants, biochemical limitation (lb) was not affected by provenance, but it was higher in plants grown at higher temperature. This provides further evidence of the potential adaptation of the biochemical apparatus of high-elevation plants to low temperatures, similar to the findings by Bunce et al. (2000) and proposed by Shi et al. (2015). On the other hand, the response of azonal plants was different and highly determined by plant provenance. While plants from

higher elevation did not show differences in their limitations when growing at different temperatures, plants from 2600 m a.s.l. exhibited lower mesophyll limitation (lm) when grown at higher temperature, accompanied by higher lb and ls. All in all, our results revealed differential responses between zonal and azonal plants, with provenance and growth temperature affecting these responses. This highlights the importance of these factors in explaining the photosynthetic and morphological patterns observed in the field.

Overall, our results indicate that temperature may not be the main responsible for the elevational changes in LMA but does have distinct effects on photosynthesis and its diffusive determinants, g_m and g_s. Additionally, the importance of the specific characteristics of plants growing at different elevations is highlighted, as these characteristics vary independently of environmental changes, such as the higher gs observed in zonal plants from higher elevation. Finally, our results underscore the differential response of zonal and azonal plants to changes in growth temperature, showing that azonal plants are more sensitive to temperature, likely due to the greater thermal stability of their water-saturated environment.

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Capítulo 3

The effects of seasonal temperature decrease on Photosynthesis in high-Andean wetland species of central Chile

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Abstract

Mountain ecosystems present significant challenges for plant photosynthesis due to fluctuating temperatures, water availability, and reduced CO_2 partial pressure at higher elevations. This study investigates the photosynthetic and leaf morphological responses of three high-Andean wetland species: *Colobanthus quitensis*, *Oxychloe andina*, and *Plantago barbata* across two elevations (2600 and 3550 m a.s.l.) and two seasonal temperatures. Gas exchange, mesophyll conductance (g_m), and leaf mass per area (LMA) were measured early and late in the growing season. We hypothesize that decreasing temperatures will result in higher LMA and lower mesophyll conductance (g_m), increasing the diffusional limitation of CO_2 and leading to reduced photosynthetic rates. Results indicate that low-elevation plants exhibit significant reductions in photosynthesis and g_m late in the season, correlating with declining temperatures. In contrast, high-elevation plants maintain stable photosynthetic rates, highlighting adaptations to cooler climates. While LMA increased across species at the end of the growing season, it showed minimal correlation with photosynthesis. Instead, g_m emerged as the primary limitation, underscoring its pivotal role in photosynthetic performance. Species-specific strategies were observed, with higher LMA buffering *O. andina* against environmental stress, while low-LMA species (*C. quitensis* and *P. barbata*) were more sensitive to thermal variability. These findings emphasize g_m as a critical factor mediating photosynthetic adaptation in Andean plants and highlight the complexity of their responses to altitude and seasonal changes.

Introduction

Mountains are considered extreme environments due to the challenging conditions present at higher elevations, such as low temperatures, reduced partial gas pressure, seasonal droughts, and short growing seasons, among others (Körner 2021). Despite this, plants are able to perform photosynthesis and thrive in these environments adjusting their functioning to cope with climatic adversities and taking advantage of favorable periods. Significant research has focused on understanding how plants respond to the environmental challenges of mountain habitats. However, disentangling the specific role of each variable on plant photosynthesis remains complex due to the multitude of factors that co-vary both spatially and temporally.

In addition to the environmental constraints observed with increasing elevations, significant variations also occur throughout the seasons, particularly during the growing season. Studies carried out with Eddy-covariance techniques indicates that seasonal climatic variations constitute a major driver of changes in carbon assimilation (Hui et al., 2003). Research in Mediterranean mountain ecosystems supports this notion. For instance, Reyes-Bahamondes et al. (2022) observed a seasonal decrease in photosynthesis in high-elevation plants (3600 m a.s.l.); however, these changes were not attributed to internal plant dynamics, such as the saturation of reserve sugars. Instead, their findings suggest that the observed changes in photosynthesis of high-elevation plants might be associated with low temperatures at the end of the growing season. In contrast, at lower elevation (1600 m a.s.l.), reduced photosynthetic rates were linked to severe water deficits. Likewise, Winkler et al. (2018) also documented a decline in plant photosynthesis over the course of the growing season, which was attributed to reduced leaf water potential and seasonal droughts. These findings indicate that temperature and water availability experienced seasonally can be critical factors shaping photosynthetic activity in mountain ecosystems.

Several studies have documented increases or decreases in plant photosynthesis attributed to temperature differences (Shi et al., 2015; Kao and Chang, 2001; Gratani et al., 2014; Ma et al., 2015; Xing et al., 2024; Zhai et al.,

2024). One proposed mechanism to explain the effects of low temperatures on photosynthesis suggests a negative correlation between temperature and the leaf mass per area ratio (LMA). Increases in LMA under lower temperatures have been observed in field measurements across various regions worldwide (Dong et al., 2020; Ogaya and Peñuelas, 2007), as well as in meta-analyses (Poorter et al., 2009). Consistently, a global correlation has been observed between higher LMA and reduced mesophyll conductance (g_m), reflecting a trade-off between resistance and resource acquisition within the leaf economic spectrum (Onoda et al., 2017). The negative correlation between g_m and LMA can be explained by an increase in leaf thickness, which increase the diffusion pathway for CO_2 to reach the chloroplasts, thereby limiting photosynthesis. Additionally, higher LMA may result from an increase in cell wall thickness, leading to greater leaf density, further constraining CO_2 diffusion in the liquid phase (Tomás et al., 2013) and determining lower photosynthetic rates per area under reduced growth temperatures.

One of the challenges in testing the effect of temperature on the photosynthesis of mountain plants lies in the altitudinal changes in the CO_2 partial pressure. Several authors have discussed the impact of reduced CO_2 partial pressure on plant photosynthesis. Wang et al. (2017) argues, based on theoretical analysis, that the reduction in photosynthesis associated with a 1000 m increase in elevation is minimal, owing to the increased gas diffusion coefficient at lower partial pressures, a concept previously introduced by Terashima et al. (1995). Conversely, Takeichi et al. (2013) demonstrated that higher gas partial pressures result in greater photosynthesis rates in *A. thaliana*, attributed to the increased CO_2 partial pressure and, consequently, a higher concentration of dissolved CO_2 in the leaf's liquid phase. In line with this, Körner et al. (1987) proposed that plants enhance their biochemical capacity at higher elevations to compensate for the reduced CO_2 partial pressure, a hypothesis supported by various field measurements (Peng et al., 2020; Guan et al., 2018). As a result, it cannot be ruled out that altitudinal differences in plant photosynthesis are influenced by the effects of partial gas pressure, as well as by plant adaptations to reduced CO_2 partial pressure. Another complexity of mountain environments is the differential responses of various genotypes (Shrestha et al.,

2019) or populations from different elevations to environmental changes, as observed in multiple studies (Melcher et al., 1977; Hernández-Fuentes et al., 2023; Viveros et al. unpublished). This highlights the value of seasonal temperature changes to examine their effects on leaves subject to the same gas partial pressure and from the same altitudinal population. Andean wetland species grow in water-saturated environments. Hence, they are not subject to the water limitations that affect other zonal species in Mediterranean mountain ecosystems. They provide an excellent opportunity to study the effects of temperature without water deficit. With this in mind, we aimed to evaluate photosynthetic and LMA changes in three wetland species of high-Andean mountain in central Chile, growing at two elevations: 2600 and 3550 m a.s.l., both, during the beginning of the growing season, when air temperatures are higher, and at the end of the growing season, when air temperatures decline (Cavieres et al., 2007; Quiroz et al., 2009). We hypothesize that decreasing temperatures will result in higher LMA and lower mesophyll conductance (g_m), increasing the diffusional limitation of CO_2 and leading to reduced photosynthetic rates. In addition, at low elevation, low temperatures could be most determinant for photosynthesis given the higher growth temperature of these plants.

Materials and methods

Study sites

The study was conducted in the central Chile Andes, at 50 km east of Santiago. Two sites at two different elevations were chosen: 2600 and 3550 m a.s.l. At both elevations three azonal species were studied: *Plantago barbata* (G. Frost Plantaginaceae), *Oxychloe andina* (Phil. Juncaceae) and *Colobanthus quitensis* (Kunth Caryophyllaceae), the species were selected according to two criteria: presence in both elevations and possibility to be measured with gas exchange devices. At lower elevation the growing season usually starts in October and ends in April. The annual precipitation is around 700 mm, predominantly falling as snow in winter (Santibáñez and Uribe, 1990). The higher elevation site presents a 4-month growing season (from December to April), and it is characterized by an average

precipitation of 943 mm, falling as snow in winter and occasionally as rain, hail, and snow during the growing season. Mean daily temperatures were extracted from Google Earth engine, from the ERA5_AG dataset between November 2021 and April 2022 at the two sampled sites. Mean day temperatures from the last 20 days before gas exchange measurements in the lower elevation site were 11.04 °C early in the season and 7.84 °C at the end of the season, while at the high elevation site, mean day temperatures were 6.73 °C in the early season and 3.88 °C at the end.

Gas exchange and chlorophyll fluorescence measurements

Gas exchange, chlorophyll fluorescence, and anatomy were measured early in the growing season between December and January 2021-2022, and late in the growing season at the end of March and the beginning of April.

Leaf gas exchange was determined simultaneously with chlorophyll fluorescence using a LI-6400XT gas exchange system (LI-COR Inc., Lincoln, NE, USA) with an integrated fluorescence chamber (Li-6400-40; LI-COR Inc.). Measurements were taken on a single leaf or groups of leaves, aiming to cover the entire area of the IRGA chamber without overlapping, following the procedure described in Sáez et al. (2017). The leaf area was determined by taking a photograph and measuring the area within the chamber using the ImageJ software (Wayne Rasband/NIH, Bethesda, MD, USA). The conditions inside the leaf chamber consisted of photosynthetically active radiation (PAR) of 1500 µmol photons $m^{-2}s^{-1}$ for all species except for *C. quitensis* which was 1000 µmol photons $m^{-2}s^{-1}$, with a 10% of blue light, relative air humidity between 40% and 60%, and leaf temperature set at 15°C given that this temperature is close to the maximum temperature during mid-day for both elevations and to be able to compare measurements from different elevations and moments of the growing season. Light response curves were performed at different light intensities ranging from 30 to 130 µmol $m^{-2}s^{-1}$ under low oxygen concentration, following the method proposed by Bellasio (2016). The reduction of ambient oxygen concentration was achieved by connecting a nitrogen cylinder to the air inlet of the gas exchange system, replacing the ambient gas

concentration with an inert one for the leaf. Corrections for CO_2 leakage of the leaf chamber were applied to all gas exchange measurements, as described in Flexas et al. (2007).

The quantum efficiency of photosystem II (PSII) was determined using the equation:

$$\phi PSII = \frac{(F'm - F's)}{F'm}$$

where F's represents the steady-state fluorescence under constant light (PPFD 1500 μmol $m^{-2}s^{-1}$), and F'm represents the maximum fluorescence obtained after a saturating light pulse of 8000 μmol $m^{-2}s^{-1}$ and 0.8 s of duration. As ϕPSII represents the number of electrons transferred per absorbed photon by PSII, the electron transport rate (ETR) can be calculated as

$$ETR = \varphi PSII \cdot PPFD \cdot \alpha\beta$$

where PPFD is the photosynthetically active photon flux density, and αβ was estimated from the parameters obtained from the low-oxygen light response curves.

Estimation of leaf mesophyll conductance

The mesophyll CO2 conductance was calculated based on the combination of gas exchange and chlorophyll fluorescence, following the method described in Harley et al. (1992):

$$g_m = \frac{A_N}{\frac{C_i - (\Gamma(ETR + 8(A_N + R_n))}{(ETR - 4(A_N + R_l)))}}$$

A_N (net photosynthetic rate) and C_i (internal CO_2 concentration) were obtained from gas exchange measurements under saturating light conditions. The rate of non-photorespiratory CO_2 evolution under light conditions (R_l) was assumed to be half of the dark respiration (R_n). The CO_2 compensation point (Γ*) was calculated considering the altitudinal effects of changes in partial pressure of oxygen according to the following equation Γ*=0.5 O/(Sc/o), where O is the partial pressure of oxygen and Sc/o is the specificity factor of RuBisCO (Farquhar et al., 1980). The determination of g_m was used to calculate Cc (the chloroplast CO_2 concentration).

Leaf mass per area ratio

At each elevation, and for each study species eight to ten leaves were collected at the beginning and the end of the growing season. The leaves were scanned, and their areas measured with the ImageJ software (Wayne Rasband/NIH, Bethesda, MD, USA). Afterwards they were dried in oven at 70°C for 48 hours to measure their dry weight and calculate the LMA dividing mass by area.

Statistical analysis

Differences between parameters during the growing season were assessed through T-test or Mann-Whitney test depending on the normality of the samples. The relationships between g_s and g_m with A_N was tested in the Sigmaplot 11.0 software (Systat software Inc. 2008) using exponential rise regression analyses according to the following equation:

$$y = \propto (1 - e^{-bx})$$

While the relationship between LMA and A_N was evaluated with simple linear regression.

We modelled the direct effects of elevation and temperature on the measured morphological and photosynthetic traits using structural equation model (SEM) performed in R studio (4.4.1) with the “Lavaan Package”. We proposed a series of interactions between our environmental variables and leaf traits and removed non significative correlations until we reached the final model. Given the relatively low number of observations and its effect over chisquare values, we used 4 fit indexes, namely, standardized root mean square residual (SRMR), root mean squared error of approximation (RMSEA), comparative fit index (CFI) and the Tucker Lewis index (TLI) to accept our model.

Results

The seasonal effect on the photosynthetic gas exchange was mostly dependent on elevation (Fig. 3.1). In *C. quitensis*, net photosynthesis (A_N) and the mesophyll conductance (g_m) were reduced at the end of the growing season only in plants growing at low elevation (2600 m a.s.l.). At higher elevation (3550 m a.s.l.) no changes were observed between the beginning and the end of the growing season. Stomatal conductance (g_s) remained constant across elevations and across growing season. *O. andina* exhibited stable photosynthetic parameters (A_N, g_s, g_m) across both elevations and throughout the growing season, except for a decrease in g_s at high elevation late in the season. *P. barbata* showed a similar pattern to *C. quitensis*, however in *P. barbata* g_s was also reduced late in the growing season in plants from low elevation.

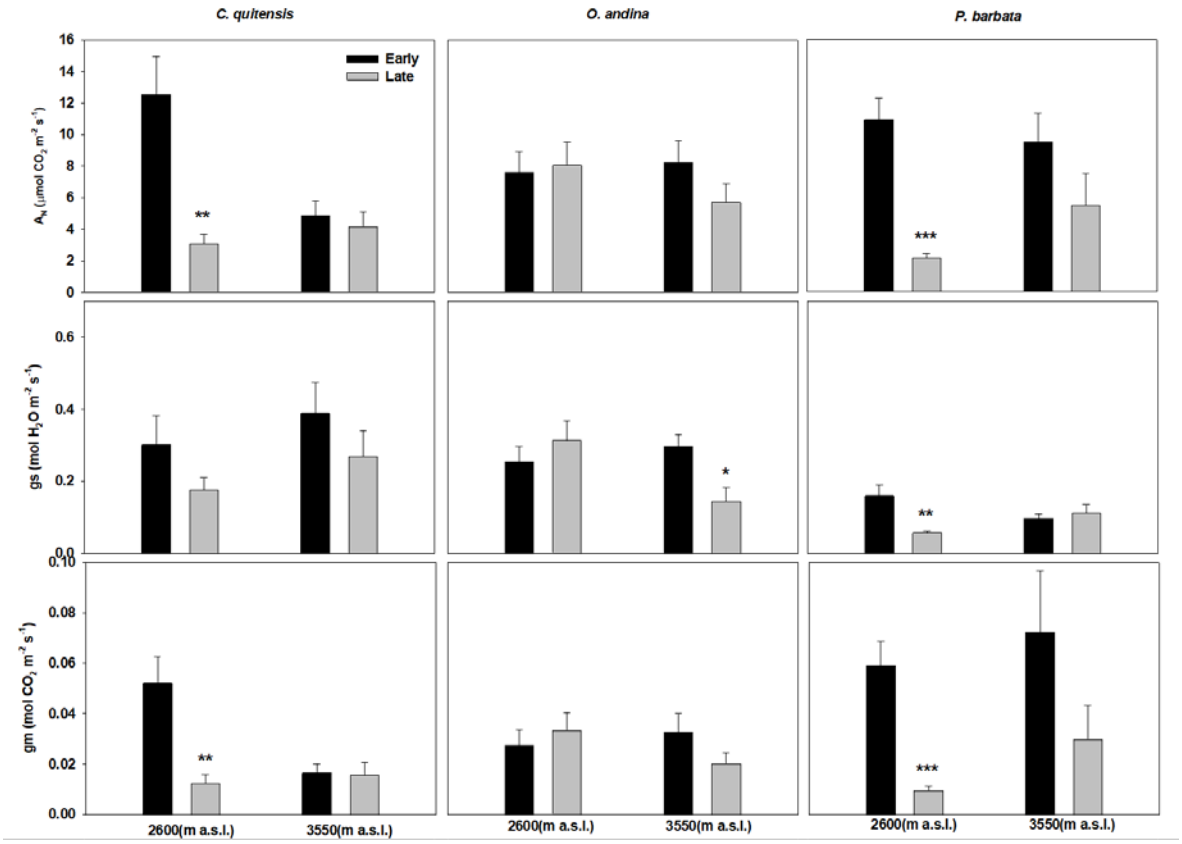

Fig 3.1. Leaf photosynthetic traits (net photosynthesis, A_N; stomatal conductance, g_s and mesophyll conductance, (g_m) in azonal species: *C. quitensis*, *O. andina* and *P. barbata* growing at 2600 and 3550 m a.s.l and measured early and late in the growing season. Values are means ±S.E. (n=6). Asterisks indicate statistically significant differences between early and late in the growing season according to T-test o Mann-Whitney test depending on the distribution of the samples and with a p value of 0.05 * 0.01** and 0.001***.

When analyzing the coordination between net photosynthesis with g_s and g_m, different patterns were observed among species and elevations (Fig. 3.2). For *C. quitensis*, when data from early and late in the growing season were pooled together, significant correlations were observed only between A_N and g_m in plant from low and high elevation. In *O. andina*, significant correlations were observed between A_N and g_s only at high elevation. On the contrary, A_N and g_m significantly correlated in plants

from both elevations. Finally, in *P. barbata*, A_N and g_s correlated only at low elevation, while A_N and g_m correlated at both elevations.

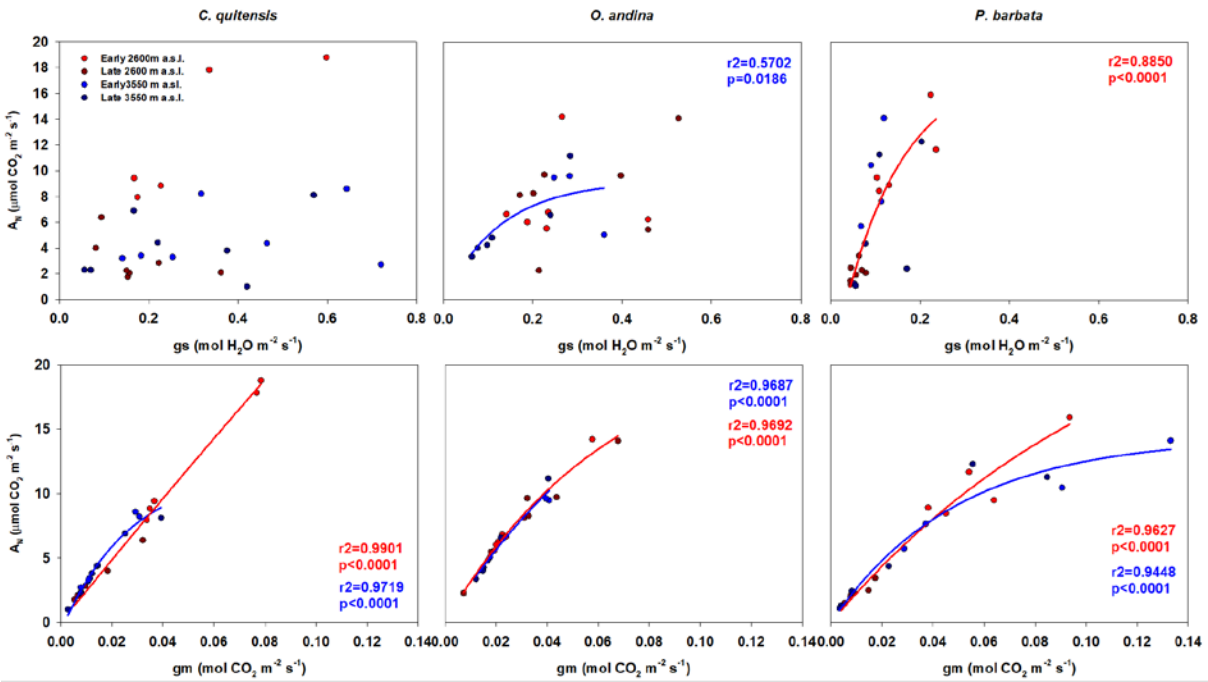

Fig 3.2. Correlations between net photosynthesis (A_N) and stomatal (g_s) and mesophyll (g_m) conductance in azonal species: *C. quitensis*, *O. andina* and *P. barbata* growing at 2600 (red) and 3550 (blue) m a.s.l and measured early and late in the growing season. In the case of a statistically significative regression, R Squared and p value from the regression analysis are presented.

For all azonal species evaluated, leaf mass area (LMA) significantly increased at the end of the growing season across both elevations (Fig. 3.3). *C. quitensis* exhibited the greatest variability, while *O. andina* showed the highest final LMA value. Notably, no correlation was found between LMA and A_N for any species (insert Fig. 3.3). In addition, mean LMA from the three species showed significative differences between them (Fig S 3.1).

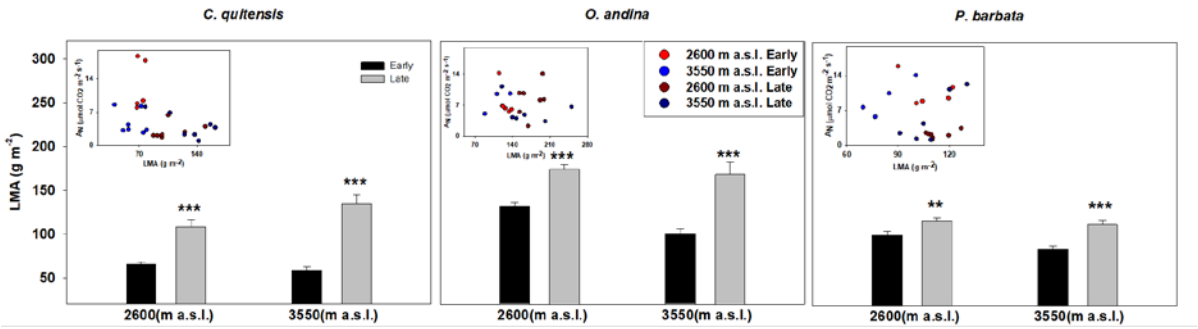

Fig 3.3. Leaf mass area ratio (LMA) in azonal species: *C. quitensis, O. andina* and *P. barbata* growing at 2600 and 3550 m a.s.l and measured early and late in the growing season. Values are means ± SE (n=9). Asterisks indicate statistically significant differences between early and late in the growing season according to T-test o Mann-Whitney test depending on the distribution of the samples and with a p value of 0.01 ** and 0.001 ***. The inner graphics show dot plots from non-significant correlations between LMA and A_N in each species and elevation.

An initial model including all species and elevations proposed a series of relations where elevation is inversely related to seasonality (temperature), which in turn determines higher LMA and lower g_m at cooler temperatures (late in the growing season). When put to test, we reached a final adjusted model in which temperature significantly affects g_m and LMA, but the impact on photosynthesis is predominantly mediated through g_m, as LMA shows a minimal correlation with A_N, Fit indexes are shown in Table S 3.1.

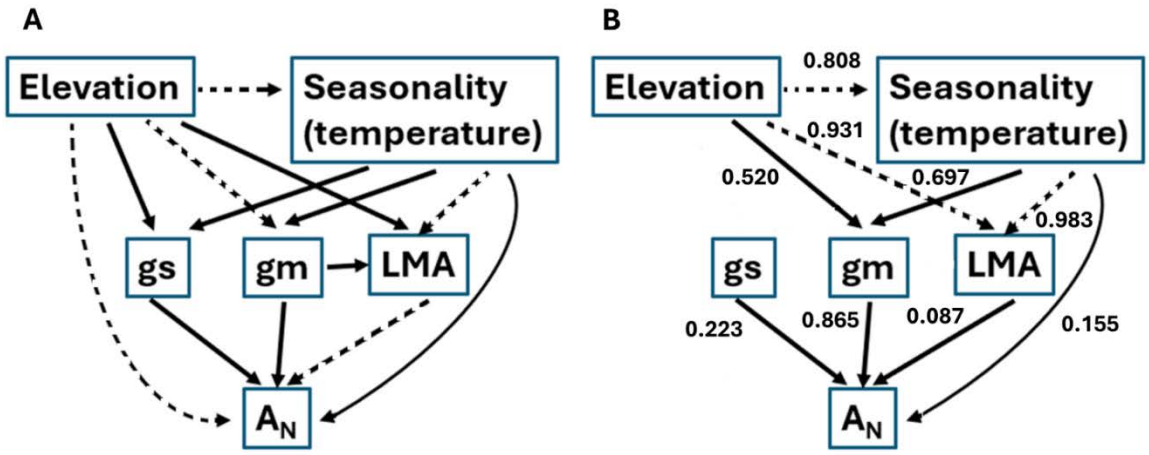

Fig. 3.4. Model for the effect of elevation and timing on the growing season (temperature decrease) over photosynthesis through leaf mass area (LMA) stomatal (g_s) and mesophyll conductance (g_m). Factor loadings are presented beside the lines. Continuous lines represent direct correlations, while dotted lines represent inverse correlations between variables. All correlations from SEM were significant ($p<0.05$).

Discussion

Our results show that plants from lower elevation are more susceptible, compared to high elevation plants, to the environmental adversities of the late of growing season, namely, lower temperatures. These results agree with the differential responses of mountain plants from different elevations when grown in common garden experiments (Hernandez-Fuentes et al., 2023) and in the field (Reyes-Bahamondes et al., 2022). The above suggests that the low temperature could be a key determinant of photosynthetic rates in low elevation azonal species, which grow at higher temperatures. Net photosynthesis in high elevation plants seem adapted to low temperatures, showing no significant changes between the beginning and the end of the growing season. However, despite these general trends, species specific responses were found. For instance, *C. quitensis* and *P. barbata*, low LMA species (Fig S 3.1), were more susceptible to thermal variability within the growing season,

when they grow at lower elevation, as evidenced by the significant reductions in photosynthesis and conductance parameters (Fig 3.1). On the contrary, the lack of response from the higher LMA species *O. andina*, could indicate adaptations that buffer against environmental stress, as proposed by the leaf economic spectrum (Onoda et al., 2017). *O. andina* was the only species that significantly decreased gs at the end of the growing season in high elevation. This could be a heat conservation strategy when temperatures start to decrease, similar to *P. barbata* at lower elevation. These results highlight the individual strategies of plants to cope with low growth temperature.

We initially proposed g_s and g_m as responsible for the A_N changes. But the low correlation between g_s and A_N, as expected, rules out g_s as responsible for the A_N response to late season lower temperatures (Fig 3.2). This may be because of the ample water supply of azonal species, which leads to higher g_s values (Viveros et al., 2024). Instead, the high correlation between g_m and A_N seems to explain photosynthetic decreases in response to low temperatures. The consistent correlation between g_m and A_N across all conditions (Fig 3.2) underlines the crucial role of mesophyll conductance as a primary limitation to photosynthesis in alpine plants (Viveros et al., 2024; Xing et al., 2024), even under the different growing temperatures experienced at different moment during the growing season. This suggests that strategies to enhance g_m could be vital in supporting photosynthesis under stress conditions.

Our hypothesis stated that g_m changes would be related to LMA changes. But the increase observed in LMA at the end of the growing season for all species studied (Fig 3.3) can result from the delaying effect that lower temperatures have on leaf development (Gunn et al., 1999; Shimono et al., 2002). Lower growth temperatures would increase the time required for leaves to reach their maximum LMA and photosynthesis values, as these traits increase during leaf development (Tosens et al., 2011). Accordingly, high elevation species seem to have lower LMA values in the early growing season but reach similar values later, similar to Vivero et al. (unpublished, Chapter 2) where no differences in LMA were found across grow

temperatures. Lower LMA at the end of the growing season could also be an adaptive response to decreasing temperatures (Onoda et al., 2017; Ogaya et al., 2007), but it does not seem to have an effect over g_m, as expected. This independence of LMA from g_m has been observed in nonlinear models, where g_m values would depend on other anatomical characteristics, such as the arrangement of chloroplasts exposed to air spaces and the thickness and composition of the cell wall (Rahimi-Majd et al., 2024; Viveros et al., 2024; Clemente-Moreno et al., 2019), which could increase independently of the LMA. Another component that has been little studied but could have a significant effect on g_m independently of LMA are aquaporins, which are capable of transporting CO_2 across lipid membranes and have been linked to the g_m of mountain plants (Xing et al., 2024) explaining changes in g_m and A_N, independently from LMA.

When analyzing all species together, the measurements presented in this study show an effect of seasonality on LMA and g_m, but not a correlation between g_m with LMA as proposed in our initial hypothesis (Fig 3.4). The direct effect of temperature on g_m appears to be the primary mechanism determining the photosynthesis of azonal high-Andean plants, with minimal contribution of LMA. This is reflected in a high factorial loading of g_m on A_N and a low factorial loading of g_s, temperature, and LMA (Fig 3.4). Also, this work shows differential elevational responses to lower temperatures. For instance, low elevation photosynthesis was more affected by late season, which is characterized by lower temperatures. LMA also showed strong seasonality which is up most important to account when sampling mountains and in general highly seasonal environments.

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Suplementary information

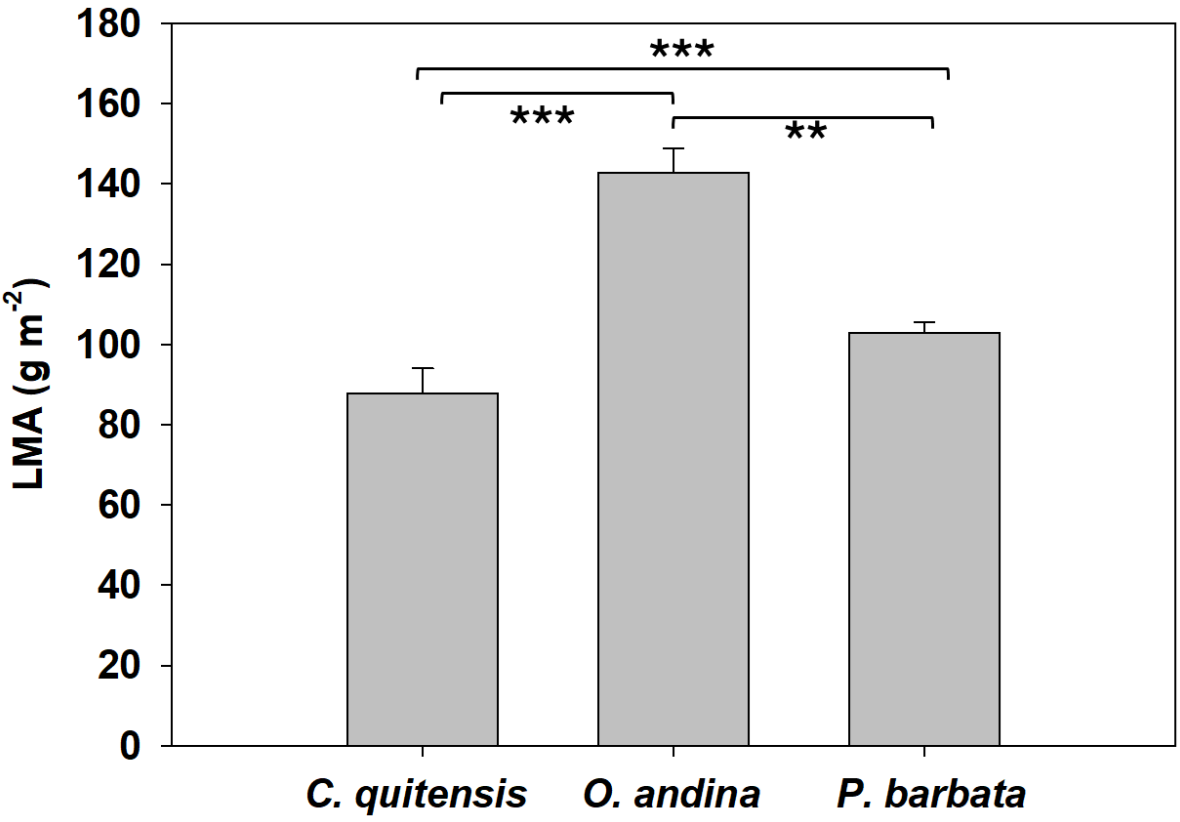

Fig S 3.1. Leaf mass area ratio (LMA) in azonal species: *C. quitensis*, *O. andina* and *P. barbata* pooled from all measurements. Values are means ± SE (n=35). Asterisks indicate statistically significant differences between species according to Mann-Whitney test with a p value of 0.01 ** and 0.001 ***.

Fit Index	Value
SRMR	0.056
RMSEA	0.083
CFI	0.990
TLI	0.973

Table S 3.1. Fit indexes of the final SEM. Final values of fit estimators SRMR, RMSEA, CFI and TLI.

Discusión general y conclusiones

Fotosíntesis y sus limitaciones

Las expectativas teóricas para la fotosíntesis predicen pocos o ningún cambio en la fotosíntesis con la altitud (Wang et al., 2017). Sin embargo, mediciones de campo han encontrado distintos patrones (Ma et al., 2015; Gratani et al., 2014; Kao y Chang, 2001; Kogami et al., 2001), los que han sido atribuidos principalmente a temperaturas más bajas y condiciones climáticas adversas a medida que incrementa la elevacion (Shi et al., 2015; Kao y Chang, 2001; Gratani et al., 2014; Ma et al., 2015; Xing et al., 2024; Zhai et al., 2024). Los cambios en la fotosíntesis pueden atribuirse a las tres principales limitaciones de A_N.

En ambientes de montaña, no se espera que las limitaciones bioquímicas sean de gran importancia, ya que algunos estudios han propuesto ajustes enzimáticos a temperaturas frías (Körner et al., 1987; Hüner y Macdowall, 1979; Yamori et al., 2006). De hecho, mediciones de campo han encontrado una mayor capacidad bioquímica con la altitud (Mehta et al., 2024; Peng et al., 2020; Körner et al., 1987; Guan et al., 2018). En nuestras mediciones, la limitación bioquímica no fue una limitante mayor en ningun caso estudiado, lo cual apoya la alta capacidad bioquímica de plantas de montaña. También, el aumento de las limtiaciones bioquimicas ante las mayores temperaturas de crecimiento, apoyan la adaptación del aparato bioquímico de plantas de montaña a las bajas temperaturas de crecimiento, especialmente en plantas zonales.

En cuanto a las limitaciones difusivas, se esperaba que la limitación estomática (ls) y la limitación del mesófilo (lm) fueran importantes en plantas zonales de baja altitud, que enfrentan sequía durante la temporada de crecimiento (Flexas et al., 2004; 2009; Galle et al., 2009; Galmés et al., 2007). En contraste, en plantas azonales, que no enfrentan limitaciones hídricas, se esperaba que la conductancia del mesófilo fuera la principal limitación, especialmente en elevaciones altas debido a las bajas temperaturas. Nuestros resultados muestran que, tanto en condiciones

de campo como en experimentos de laboratorio, la conductancia del mesófilo es la principal limitación para la fotosíntesis en plantas de montaña. Sin embargo, la mayor g_s proporcional en plantas azonales parece aumentar la lm en este grupo. Por otro lado, la ls mostró ser importante solamente en plantas zonales cuando co-limita la fotosíntesis junto con la conductancia del mesófilo, probablemente debido a la menor presión parcial a mayor elevación combinado con las menores temperaturas de crecimiento.

En relación con los mecanismos detrás de las variaciones de A_N, trabajamos con la hipótesis de que condiciones ambientales más adversas aumentarían el LMA debido a un mayor grosor de la pared celular y un aumento en la trayectoria de difusión hasta el sitio de carboxilación dentro del cloroplasto (Onoda et al., 2017). Las mediciones de campo y laboratorio muestran que A_N tiene poca correlación con LMA, a pesar del fuerte aumento estacional de los valores de LMA al final de la temporada de crecimiento, cuando las temperaturas son más bajas. El aumento del LMA al final de la temporada de crecimiento puede resultar del efecto de retraso que las temperaturas más bajas tienen sobre el desarrollo de las hojas (Gunn et al., 1999; Shimono et al., 2002). Temperaturas de crecimiento más bajas aumentarían el tiempo necesario para que las hojas alcancen sus valores máximos de LMA, ya que estos rasgos aumentan durante el desarrollo foliar (Tosens, 2011). En consecuencia, las especies de mayor elevación parecen tener valores de LMA más bajos al inicio de la temporada, pero alcanzan valores similares al final de la misma, esto concuerda con los resultados de experimentos de cámara donde no se encontraron diferencias en LMA en función de la temperatura de crecimiento. Un LMA más alto al final de la temporada de crecimiento también podría ser una respuesta adaptativa a la disminución de temperaturas (Onoda et al., 2017), aunque no parece tener un efecto sobre g_m , como se esperaba.

En cambio, la g_m estaría influenciada principalmente por la disposición de los cloroplastos, similar a los hallazgos de otros estudios en plantas antárticas, helechos y briofitas (Sáez et al., 2017; Carriquí et al., 2019) o cálculos teóricos donde se ha atribuido el 50% de la resistencia a la difusión de CO2 a los cloroplastos

(Tholen et al., 2012). También, el grosor de la pared celular mostró correlación con g_m en plantas zonales y especies de menor elevación al igual que en otros trabajos (Terashima et al., 2011; Tomás et al., 2013). Nuestros resultados están en línea con estudios recientes que encuentran en gramíneas C3 una fuerte relación entre g_m y la disposición de los cloroplastos, así como con las características de la pared celular (Rahimi-Majd et al., 2024; Clemente-Moreno et al., 2019). Nuestros resultados recalcan la importancia de los factores microanatómicos independientemente del LMA como determinantes de la conductancia del mesófilo.

La falta de correlación entre LMA y características anatómicas que si han sido observadas en otros estudios (Tosens et al., 2011; Tomás et al., 2013) podría deberse a que estas características pueden ser importantes determinantes del LMA pero no correlacionar con este (John et al., 2017). Por ejemplo, Las hojas con mayor LMA de mayor elevación pueden tener una mayor fracción de pared celular sin que esta sea mas gruesa, si el número de células del mesófilo es mayor y su tamaño menor, lo que aumentaría el área de pared celular por área de hoja. También, la composición de la pared celular ha demostrado importantes efectos sobre la conductancia del mesófilo independiente del grosor de la pared celular, a través de una expresión diferencial de pectinas y otros metabolitos del apoplasto (Clemente-Moreno et al., 2019). También, los altos valores de LMA encontrados a final de temporada no mostraron relación con los valores de gm, esto podría explicarse por un mayor número de capas de células del mesófilo y un valor constante de nitrógeno por área destinado al aparato fotosintético (Jhon et al., 2017). Debido a esto los componentes del LMA podría tener distintas implicancias en las características fotosintéticas de las hojas si se analiza en detalle.

Los resultados de esta tesis muestran cómo los patrones fotosintéticos altitudinales pueden estar determinados por la respuesta particulares de las distintas poblaciones a la temperatura de crecimiento, similar a los hallazgos de Hernandes et al. (2023) en *Phacelia secunda*. Asimismo, cuando se evalúan a la misma altitud, las plantas azonales de menor altitud muestran grandes disminuciones estacionales en la fotosíntesis, mientras que las plantas de mayor

altitud mantienen tasas fotosintéticas estables durante la temporada de crecimiento. Estos resultados dan cuenta de que diferencias altitudinales en parametros fotosintéticos pueden estar determinados altitudinalmente independiente de las condiciones ambientales. Por ejemplo, plantas zonales de mayor elevación tienen mayores valores de gs que plantas de menor elevación a pesar de crecer en las mismas condiciones (jardín común).

Efecto de la temperatura sobre la fotosíntesis

El efecto de la temperatura sobre los parámetros fotosintéticos parece ser complejo. Por un lado, los experimentos de laboratorio mostraron una mayor fotosíntesis y gm a menores temperaturas diurnas de crecimiento. Un factor importante que podría ayudar a explicar este efecto es el hecho de que a bajas temperaturas la especificidad de la RuBisCo es mayor hacia el CO_2 en desmedro del O_2 (John et al., 2017) o las adaptaciones antes mencionadas del aparato bioquímico a las bajas temperaturas, como mayor cantidad de RuBisCo o una menor temperatura óptima de las enzimas involucradas en la fotosíntesis (Huner & Macdowall 1979; Yamori et al. 2006). Por otro lado, en nuestras mediciones de campo, encontramos menores tasas de fotosíntesis en plantas de menor elevación expuestas a menores temperaturas estacionales. Cabe destacar que estas respuestas opuestas podrían deberse a las grandes variaciones de temperatura que experimentan las plantas en terreno, comparado con las estables condiciones de los experimentos de laboratorio. El posible efecto negativo de la temperatura sobre la gm podría deberse, independientemente del LMA, a la disminución del coeficiente de difusión de CO2 en el medio acuoso, el cual disminuye en 30% con un una disminución en 10°C de temperatura (Tholen et al., 2012). Las bajas temperaturas, también podría afectar las propiedades bioquímicas de las acuaporinas, proteínas de membrana que han demostrado ser transportadores de CO_2 y estar correlacionada su expresión con la conductancia del mesófilo (Xing et al., 2024; Flexas et al., 2007). Estos antecedentes podrían explicar también los bajos valores de gm y alta lm encontrada en las plantas de alta montaña, las cuales fueron medidas a 15°C en terreno.

Plantas zonales y azonales

Como se esperaba, plantas zonales y azonales mostraron marcadas y variadas diferencias en sus respuestas a la elevación y a la temperatura. Por ejemplo, las plantas azonales mostraron menor LMA y A_N con la elevación, mientras que plantas zonales mostraron similares valores a distinta elevación. También, plantas zonales y azonales mostraron diferencias en sus limitaciones a la fotosíntesis, de acuerdo con las expectativas, la limitación estomática resultó ser más importante en plantas zonales. La baja limitación estomática de plantas azonales debido a la alta presencia de agua en sus ambientes parece aumentar la importancia de la limitación por conductancia del mesófilo en estas plantas comparado con plantas zonales. Finalmente, debido a la mayor estabilidad térmica ambiental observada en plantas azonales (Ruthzatz et al., 1978), se aceptó nuestra hipótesis de mayor sensibilidad a los aumentos de temperatura en plantas azonales. Las especies azonales mostraron disminuciones en g_s y g_m ante las mayores temperaturas de crecimiento, mientras que plantas zonales no mostraron respuesta en estos parámetros. A través de distintas evidencias, nuestros resultados recalcan las marcadas diferencias en las respuestas de plantas zonales y azonales a la elevación y la temperatura.

Gracias a este análisis exhaustivo, el presente estudio no solo contribuye a nuestra comprensión de la fisiología vegetal, sino que también mejora nuestro conocimiento sobre cómo los ecosistemas de alta montaña pueden funcionar en respuesta a sus condiciones ambientales. También, esta investigación ofrece valiosos conocimientos sobre los procesos fundamentales de adaptación de las plantas a grandes altitudes, destacando el papel crítico de la conductancia mesofílica. Establece las bases para futuros estudios ecológicos y fisiológicos, con el objetivo de dilucidar las complejas interacciones entre la morfología vegetal, los rasgos fisiológicos y los factores ambientales en las regiones montañosas. Las implicaciones de estos hallazgos son significativas para contribuir a predecir las respuestas de la vegetación alpina a los cambios ambientales y para la conservación y gestión de estos ecosistemas sensibles. Esta tesis proporciona evidencia de cómo g_m depende de los rasgos anatómicos, en particular de la disposición de los cloroplastos, en lugar de depender del LMA, como se ha propuesto en otros estudios. También, deja en manifiesto la respuesta fotosintética

diferencial de las plantas zonales y azonales a distintas condiciones ambientales, como la altitud, el momento de la temporada de crecimiento y la temperatura de crecimiento. Además, este trabajo muestra que las respuestas fotosintéticas y de la g_m están determinadas por el origen altitudinal de las plantas, lo cual es crucial para interpretar los patrones espaciales de la fotosíntesis. Finalmente, se resalta la importancia de la g_m como la principal limitación de la fotosíntesis en plantas de montaña, tanto zonales como azonales.

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