Eco-physiological responses of *Hieracium pilosella* and *Trifolium pratense* to reduced air pressure

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Short title: Eco-physiological response to reduced air pressure

Abstract

Climate change is a major factor shaping the distribution of plant species. A well-documented response consequence is the upward shift of plant species to higher elevations as they track their thermal niches. However, plants migrating upward face complex environmental changes shaped by multiple interacting factors. Among these, reduced air pressure remains relatively understudied, its effects are often confounded with other covarying parameters. This study investigated the direct impact of reduced air pressure on the eco-physiological responses of two plant species (*Hieracium pilosella* L. and *Trifolium pratensis* L.). The plants were grown for four weeks in controlled climatic chambers under different air pressures (85, 75, and 62 kPa), while all other environmental

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parameters were kept constant. At the end of the experiment, photosynthesis, chlorophyll fluorescence, growth, carbohydrate content, carbon stable isotopes, and plant nitrogen concentrations were determined. Reduced air pressure decreased growth, carbon isotopic discrimination and chlorophyll content, but increased CO_2 fixation efficiency and carbohydrate accumulation in the leaves. These results suggest that reduced air pressure impacts plant performance during upslope migration and may, in turn, contribute to shaping future distribution patterns in alpine ecosystems.

Keywords: alpine area, air pressure, carbon dioxide, growth, *Hieracium pilosella*, oxygen, *Trifolium pratense*

Abbreviations: C, carbon; NSC, non-structural carbohydrates; C_i, intercellular CO_2 concentration; C_a, ambient CO_2 concentration; A, photosynthesis rate; gs, stomatal conductance; N, nitrogen; $\Delta^{13}C$, carbon isotope discrimination; CN ratio, carbon to nitrogen ratio; RGR, relative growth rate; SLA, specific leaf area; LDMC, leaf dry matter content; PAR, photosynthetically active radiation; RH, relative humidity; ANOVA, analysis of variance. ANCOVA, analysis of covariance; $\rho\rho O_2$, partial pressure of O_2; $\rho\rho CO_2$, partial pressure of CO_2; PPFD, photosynthetic photon flux density; Photosystem II, PSII; photochemical efficiency of PSII, ϕPSII; F_v/F_m potential efficiency of PSII, *NPQ*, non-photochemical quenching; VPD, vapor pressure deficit

1. Introduction

Alpine ecosystems are experiencing high warming rates due to climate change (Nigrelli and Chiarle, 2023). Warming prompts plant species to shift their distribution ranges upslope (Chen et al., 2011), enabling them to track their ecological niches and evade rising temperatures (Pauli et al., 2012). The shift in the distribution of some species to higher elevations could imply broad changes ranging from the physiological responses of individuals to alterations in ecosystem functioning (der Putten, 2012). In fact, plant species will be exposed to new environmental conditions and experience new species interactions. The responses of plant species to new environmental and geographical constraints and the establishment of new interactions with other species will trigger the appearance and development of novel traits that are likely to be of decisive importance in their adaptation process, distribution ranges, and survival.

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The impact of climate change on the biodiversity and distribution shifts of living species is a hot topic in ecological studies. The extent of habitat shifts depends on the inherent characteristics of plant species and their tolerance to environmental changes. In harsh environmental conditions, plant species with slow growth patterns dominate. These species adopt a conservative life strategy, enhancing their ability to tolerate adversity and survive in challenging environments. Grime (1979) classified such species as 'stress-tolerators,' which can store more photo-assimilates and endure long dormant periods. Slow-growing species have low survival demands and are well-adapted to impoverished environments. They operate near their optimum growth rates, allocate fewer resources to structural components, and are characterized by reduced height, low respiration rates, limited water movement, low nutrient concentrations, and a greater investment in dense tissues (Chapin, 1980).

Alpine ecosystems, where extreme environmental conditions prevail, are marked by low temperatures, short growing seasons, prolonged snow cover, high UV irradiance, intense winds, reduced air pressure, and limited nutrient and water availability (Billings, 2000). Plant species in these ecosystems have developed various physiological, anatomical, and morphological adaptations to survive these stresses, a subject of extensive research (e.g. Sati et al., 2024). While these adaptations are crucial, the primary ecological responses of plants in such environments are often attributed to low temperatures (Shi et al., 2006).

Low air pressure is one of the least studied environmental factors in mountainous regions (Jacobsen, 2020). Unlike temperature, air pressure does not decrease uniformly with elevation; it is also influenced by variations in air temperature and water vapor content. As total air pressure declines, the partial pressures of all atmospheric gases, including CO_2 and O_2, also decrease. This reduction in the partial pressures of key gases can have significant implications for plant physiology, particularly in relation to processes such as photosynthesis and respiration. Specifically, a decrease in theCO_2 availability can negatively impact photosynthesis, as CO_2 is a crucial raw material for this process. Conversely, a decrease in the availability of O_2 could have a positive effect due to a reduction in the photorespiration rate. However, gas diffusion increases under reduced air pressure conditions, which positively affects photosynthesis by increasing the stomatal conductance of leaves. These opposing effects can compensate for each other (Terashima et al., 1995), with the net outcome depending on the photosynthetic capacity of the plant species within a specific ecosystem.

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Previous studies have reported diverse rates of leaf gas exchange at high elevations (Cordell et al., 1999; Shi et al., 2006).

Furthermore, the role of carbon (C) limitation in constraining plant growth at high elevations remains insufficiently understood and requires further clarification. It is widely known and evidenced that plants fix C at a higher rate than needed in lowland regions (e.g., Hermans et al., 2006) and some authors suggest that there is no C limitation at a high elevation (e.g. Möhl et al., 2020). Furthermore, in the context of global change, the atmospheric CO_2 rate is increasing faster than the average rate of the upward migration process, thus compensating for any decrease in CO_2 availability to migrating plants. Thus, any expected C limitation for plants at high elevations may be related to species-specific traits, such as stress tolerance, storage capacity, and symbiosis with microorganisms in the soil. The trade-off between stress tolerance and growth rate is also important in assessing the C budget of plant species at high elevations. Therefore, studies focusing on the direct effects of reduced air pressure on plant physiology can provide insights into the adaptation and evolution of plant species in alpine regions.

Low air pressure is the missing piece for gaining a deep understanding of the physiology of plant species inhabiting alpine regions. Investigating the effect of hypobaric pressure on plant physiology is crucial to understanding upslope adaptation (Frei et al., 2014) and optimizing reduced-pressure systems for extraterrestrial plant cultivating (Paul and Ferl, 2006). While reduced air pressure is not a driver of plant migration, it may act as a physiological constraint that limits the success of upward range shifts under climate change. In this basic study, we contribute to this knowledge by isolating the direct effects of reduced air pressure on the ecophysiology of two herbaceous species, explicitly controlling for covarying environmental parameters to avoid confounding influences. Although multiple environmental factors interact to shape a species' eco-physiological response in natural habitat, our approach focuses on disentangling the specific contribution of reduced air pressure.

Given that plants respond differently to novel atmospheric conditions likely due to differences in physiological tolerances and adaptability, we selected two plant species (a forb and a legume) both common along broad elevational gradients in the Alps to compare the magnitude of the effects of reduced air pressure on plant physiology and morphology. Plants were collected from the natural habitat and transplanted into new pots containing bulk soil, following established methodologies (e.g. Midolo et al., 2020; Ali and Vyas, 2025). Transplant experiments provide tool for investigating how plant species might shift their distributions in response to climate change (Lee-Yaw et al., 2016).

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By simulating plant responses across environmental gradients, these experiments offer important insights for ecological forecasting under global change (Dainese et al 2024). The experiment was conducted using Ecotron chambers set at air pressures of 85, 75, and 62 kPa, corresponding approximately to 1500, 2500, and 4000 m above sea level (a.s.l.), respectively. The objectives of this study are: i) to isolate and evaluate the effects of reduced air pressure on the physiology of two plant species, *Trifolium pratense* L. and *Hieracium pilosella* L., by assessing leaf gas exchange, chlorophyll fluorescence, growth, non-structural carbohydrates, carbon stable isotopes, and nitrogen content; and ii) to determine the extent to which reduced air pressure acts as an abiotic stressor for mountain species, and to quantify its potential role in influencing the performance of plants migrating to high elevations.

2. Results

2.1. Growth analysis

Our results revealed a significant negative impact of reduced air pressure on the plant biomass of *T. pratense*, which exhibited a pronounced reduction in growth at 62 kPa (corresponding to 4000m a.s.l.) (Fig. 1). Reduced air pressure had a significant effect on belowground biomass but not on aboveground biomass (Table 1). In addition, the root to shoot ratio decreased significantly under reduced air pressure (Fig. 2, Table 1). In contrast, the decrease in plant biomass for *H. pilosella* was associated with significant decrease in both shoot and root biomass, while its root to shoot ratio remained unchanged (Fig. 1, Fig. 2, Table 1). When accounting for initial plant size of the plants, relative growth rate (RGR) followed the pattern observed for plant biomass. RGR decreased significantly under reduced air pressure in both targeted species (Tables 2 and 3). However, SLA and LDMC showed no significant variation with air pressure in either studied species (Tables 2 and 3).

2.2. Leaf gas exchange

In *H. pilosella*, the photosynthetic rate did not differ significantly between 85 and 75 kPa but decreased significantly at 62 kPa. A Similar pattern was observed for stomatal conductance (gs), which remained stable between 85 and 75 kPa and declined significantly at 62 kPa. In, *T. pratense,* photosynthetic rate and stomatal conductance were unaffected by reduced air pressure, although a marginally significant increase was observed between 85 and 75 kPa (p = 0.06) (Fig. 3). The ratio of intercellular to ambient partial pressure of CO_2 (C_i:C_a) decreased significantly with air pressure in *T.*

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pratense, whereas in *H. pilosella*, it decreased significantly at 75 kPa but showed a marginal increase at 62 kPa (Fig. 4).

The response of the photosynthetic rate to the increased intercellular partial pressure of CO_2 in *T. pratense* leaves was lower at 85 than at 62 kPa (air pressure F (1,37) = 12.4, p = 0.001, ANCOVA) (Supplementary material Fig. S1).

2.3. Carbon isotope discrimination ($\Delta^{13}C$)

Air pressure had a significant effect on C isotope discrimination ($\Delta^{13}C$) in both species, with a more pronounced effect in *T. pratense*. In this species, $\Delta^{13}C$ decreased significantly (less negative values) between 85 and 62 kPa in both leaves and roots. In *H. pilosella*, a significant decrease was observed between 75 and 62 kPa. Furthermore, $\Delta^{13}C$ differed significantly between the two species, with *H. pilosella* showing less discrimination against ^{13}C compared to *T. pratense*. A significant difference in $\Delta^{13}C$ between roots and leaves was also noted, with roots displaying less discrimination against ^{13}C than leaves in both species (Table 4, Fig. 5).

2.4. Chlorophyll fluorescence and chlorophyll and nitrogen content

In *T. pratense*, the photochemical efficiency of PSII (ϕPSII), the potential efficiency of PSII (F_v/F_m), and the fraction of energy dissipated as heat (*NPQ*) were unaffected by reduced air pressures in *T. pratense* plants. However, *H. pilosella* exhibited a significant decrease in ϕPSII under reduced air pressure (Tables 1–3).

The chlorophyll content remained stable between 85 and 75 kPa but decreased significantly at 62 kPa, in both species (Tables 2 and 3). Species comparisons showed higher chlorophyll content in *H. pilosella* than in *T. pratense* at 85 and 75 kPa, whereas no differences were observed at 62 kPa. The

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leaf N concentration based on dry weight did not vary across the air pressures in either species (Table 4) whereas the leaf N concentration based on leaf area decreased in *H. pilosella* between 85 and 75 kPa (Table 2).

2.5. Non-structural carbohydrates (NSCs)

Reduced air pressure had a significant effect on total non-structural carbohydrates (NSC) content, with responses varying by plant organ and plant species. In *T. pratense,* leaf NSCs increased at 62 kPa compared to 75 and 85 kPa, while root NSC decreased significantly at 62 kPa. In *H. pilosella*, leaf NSC also increased significantly at 62 kPa, while root NSC was unaffected by reduced air pressure (Fig. 6, Table 4).

3. Discussion

The upward shift of plant species to track their thermal niches in response to global change implies changes in plant physiology as they face new environmental conditions along the elevational gradient. The aim of this study was to determine the direct effect of reduced air pressure on plant physiology and performance. Our results confirm that reduced air pressure can be considered a further abiotic stress factor for plants migrating to higher elevations. This effect encompasses the associated shifts in the partial pressures of oxygen ($\rho\rho O_2$) and carbon dioxide ($\rho\rho CO_2$), which are integral components of the reduced air pressure environment, as well as changes in the $\rho\rho O_2/\rho\rho CO_2$ ratio. Although this ratio positively influences Rubisco activity, the impact of decreased $\rho\rho O_2$ on photosynthesis has been previously reported (He et al 2007, 2013). Furthermore, reduced air pressure significantly affected plant growth, $\Delta^{13}C$, and the NSC content, although the magnitude of these effects differed between species.

3.1. Plant-specific growth response to reduced air pressure

The growth rate after four weeks was significantly reduced under reduced air pressure in both species consistent with findings from a preliminary study (Lembo et al 2025), indicating that reduced air pressure affected the plants' ability to grow and/or the efficient use of photo-assimilates. This finding aligns with previous studies that have reported a delay in growth under reduced air pressure, a secondary effect observed in tomato plants (Daunicht and Brinkjans, 1996; He et al., 2007; Iwabuchi et al., 1996).). The negative impact on growth vigor under reduced air pressure could be attributed to the low $\rho\rho O_2$ which has been shown to particularly impair root development (Paul et al., 2017; He et

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al., 2007) as discussed later, or to high VPD (Iwabuchi et al 1996). Recent studies have identified high VPD as a significant constraint on plant growth (Grossiord et al., 2020), likely due to its negative effects on phloem transport and photosynthetic efficiency, which reduce carbohydrate availability for growth sinks. However, our results did not reveal a significant decline in gas exchange rates, particularly in *T. pratense*, suggesting that under our experimental conditions, the elevated VPD associated with reduced air pressure did not substantially affect photosynthetic performance. This apparent resilience may be explained by the insulating effect of the leaf boundary layer. As described by Jarvis and McNaughton (1986), a thickened boundary layer can decouple the leaf surface microclimate from ambient air conditions. In our setup, the combination of reduced air pressure and stagnant air likely enhanced boundary layer thickness, thereby buffering the leaves from external VPD fluctuations and mitigating their physiological impact.

On the other hand, the accumulation of NSC in leaves at 62 kPa suggests that growth of plant species may not have been limited by carbon under our experimental conditions. Previous studies have shown that alpine plants at higher elevations are carbon-saturated at current atmospheric CO_2 concentrations (e.g. Möhl et al., 2020). Beyond a decrease in sink demand, NSC accumulation in leaves can also be attributed to a possible effect of reduced air pressure on the ability of meristematic tissues to divide, expand, and differentiate.

Furthermore, the relative growth rate (RGR) decreased under reduced air pressure in both *H. pilosella* and *T. pratense*, with *H. pilosella* showing lower RGR values than *T. pratense*. RGR, which accounts for the initial size of individuals, allows for the comparison of plant species on a relative scale (Ruiz-Benito et al., 2015). The limited effect of reduced air pressure on *T. pratense* (p = 0.08), compared to *H. pilosella* may be partly attributed to its more developed root system, which likely stored higher levels of resources. This root system likely plays a crucial role in the plant's performance, growth, and vigor. Previous studies (e.g. Vilela et al., 2016) have highlighted the importance of stored resources from the previous year in supporting current plant growth rate. Additionally, an enhanced root system may improve symbiotic relationships with soil microorganisms and boost nutrient absorption and the assimilation *capacity* for water and *nutrients (Griffiths et al, 2022)*.

However, the slow growth characteristic of *H. pilosella* may confer an advantage under adverse environmental conditions, allowing this species to mitigate other effects of decreased air pressure. In contrast to *T. pratense*, where NSCs were depleted to meet the energy demands for root growth and respiration at reduced air pressure, the NSC concentration in *H. pilosella* roots remained

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unaffected by air pressure. This suggests that the slow growth behavior of *H. pilosella* enhances its resistance to challenging environmental conditions, leading to a lower demand for soil resources and allocation of root reserves. As a result, *H. pilosella* maintained the root to shoot ratio compared to *T. pratense*.

Furthermore, no significant effects of reduced air pressure on SLA or LDMC were observed in *T. pratense* or *H. pilosella*. Changes in these traits usually indicate plant responses to altered light and/or temperature conditions. Some studies, such as Körner et al. (1989), have reported a higher LDMC at high elevations, potentially in response to the effects of low temperature and/or low partial pressure of O_2 ($\rho\rho O_2$) (He et al., 2007). In our study, however, natural variability within each species, since plants were collected from their natural habitats, could have masked the effect of reduced air pressure on leaf traits.

3.2. Impact of reduced air pressure on photosynthetic activity

Reduced air pressure decreases CO_2 availability in the air by reducing the partial CO_2 pressure ($\rho\rho CO_2$). However, plants mitigate this effect by enhancing stomatal conductance to counteract potential C limitation and/or by increasing the efficiency of CO_2 fixation through the photosynthetic apparatus. Goto et al. (1996) showed an increase in the photosynthetic rate of spinach and maize in response to a decrease in total air pressure (from 100 to 10 kPa) while maintaining a constant $\rho\rho CO_2$. This was explained by the fact that the boundary layer and stomatal resistance to CO_2 transfer decreased at reduced air pressure, leading to a higher CO_2 diffusion coefficient, which positively affected the photosynthetic rate (Terashima et al., 1995).

Our results showed no effect of reduced air pressure on the photosynthetic rate or stomatal conductance in *T. pratense*, as already observed in previous studies (e.g., He et al., 2007). The photochemical efficiency of PSII (ϕPSII) also did not exhibit any variation. However, the C_i:C_a ratio decreased significantly with air pressure. The C_i:C_a ratio was approximated as 0.8 for C_3 species and provides information about the limitations imposed by stomatal conductance and Rubisco activity on photosynthesis. Our observed decrease in the C_i:C_a ratio indicates an increase in Rubisco demand for CO_2 exceeding the supply rate, especially since C_i decreases more than C_a with air pressure, as previously documented by Körner and Diemer (1987). The highly efficient CO_2 fixation in *T. pratense* was not attributed to higher N availability in the leaves, as the leaf N concentration based on leaf area remained consistent across the different air pressures. However, the photosynthetic response to C_i (A-C_i curves)

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showed an increase in both the slope of the curve (indicating enhanced C fixation efficiency) and the maximal photosynthetic capacity under reduced air pressure.

In *H. pilosella*, we observed a decrease in the photosynthesis rate at 62 kPa, whereas the C_i:C_a ratio decreased at 75 kPa but showed no significant variation at 62 kPa. The lack of a significant decrease in C_i:C_a at 62 kPa, despite the significant decrease in the stomatal conductance, can likely be attributed to the decline in the sink demand and photosynthate accumulation, leading to the downregulation of the photosynthetic rate and the stabilization of the C_i:C_a ratio. This is in line with the decrease in the ϕPSII observed at 62 kPa. Furthermore, the fact that reduced air pressure did not affect F_v/F_m (Table 2) indicates that the decrease in the photosynthesis rate is not due to an impairment in the photosynthetic apparatus.

3.3. Isotopic discrimination of carbon ($\Delta^{13}C$) and reduced air pressure effects

Carbon isotope discrimination, an integrative measure of plant gas exchange over a defined period, decreased along the elevation gradient. This increase in the abundance of ^{13}C aligns with findings from a previous study conducted in a natural environment (Körner, 1988). The ^{13}C abundance is intricately linked to the ratio of the internal to atmospheric CO_2 concentration (C_i:C_a ratio). The increase in the ^{13}C abundance indicates an enhancement in carboxylation efficiency, which can be attributed to the decrease in photorespiration resulting from the decreased $\rho\rho O_2$ under reduced air pressure conditions. Previous research has demonstrated a linear decrease in photorespiration with the reduction of atmospheric O_2 concentration, as O_2 is essential for generating glycolate during photorespiration (Akita, 1976).

Furthermore, the observed increase in the abundance of ^{13}C along the elevation gradient was about 0.5 and 0.33‰ km^{-1} in *T. pratense* and *H. pilosella*, respectively. This air pressure-related increase in ^{13}C abundance is slightly lower than that reported in field studies, which ranges from 0.8 to 1.2‰ per 1000 m of elevation (Zhou et al., 2011). We hypothesize that part of the reduced discrimination against ^{13}C can be directly attributed to the effect of reduced air pressure, independently of temperature or moisture effects. This effect is likely due to the low $\rho\rho O_2$ at higher elevations (Berry et al., 1972). The remaining decrease in ^{13}C isotopic discrimination observed in field studies could be linked to humidity and/or low air temperature. Low temperatures, in particular, have been shown to

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suppress photorespiration more than carboxylation, thus contributing to a further reduction in $\Delta^{13}C$ (Terashima et al., 1995).

However, the $\Delta^{13}C$ values were more negative in the leaves than in the roots in both species (about 0.4‰). The difference in $\Delta^{13}C$ between the organs of the plants has already been reported in several studies (e.g. Ghashghaie and Badeck, 2014) and could be due to an opposite respiratory fractionation between leaves and roots (Bathellier et al., 2008). Independent of the effect of reduced air pressure, the higher ^{13}C abundance obtained in *H. pilosella* compared to *T. pratense* could be attributed to its leaf anatomy characterized by thicker leaves with more mesophyll cells and a larger intercellular air space. Mesophyll conductance, which is strongly influenced by leaf anatomy (Syvertsen et al., 1995) is likely a key factor determining the ecotypic component of the ^{13}C signal in plant species (Körner et al., 1991).

3.4. Possible impact of O_2 deficiency on plant growth under reduced air pressure

Chlorophyll content decreased in both species with reduced air pressure as was observed in a previous study (Lembo et al 2005). The absence of a corresponding decrease in leaf N concentration based on leaf area suggests that the reduction in chlorophyll content is not due to nitrogen limitation, but rather likely caused by O_2 deficiency. In addition to the low $\rho\rho O_2$ in the air, the diffusion of O_2 into the soil might be slower under conditions of reduced air pressure. The chlorophyll synthesis pathway, specifically tetrapyrrole synthesis, has been shown to depend on ambient O_2 at several stages (Abbas et al., 2022). O_2 deficiency may also be responsible for the reduced growth rate observed at 62 kPa in both studied species, especially affecting root growth. Roots are known to be particularly sensitive to reduced $\rho\rho O_2$ (He et al., 2007; Paul et al 2017) due to their high dependence on oxygen for mitochondrial energy production, given their heterotrophic metabolism (Mustroph et al., 2014).

The reduced growth observed in vascular plants under O_2 deficiency could explain the observed accumulation of NSC in the leaves of both species at 62 kPa. In a study of plant tolerance to O_2 deficiency under submerged conditions, Nakamura et al. (2020) found a decrease in metabolic activities, growth cessation, and enhanced carbohydrate storage. Plants can stop growing and maintain carbohydrate reserves under stressful conditions (Fukao et al., 2006). O_2 deficiency may lead to a decline in ATP production through respiration, causing the energy generated to be allocated toward plant maintenance rather than meeting growth requirements. An additional adaptive mechanism may involve an increase in mitochondrial

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density per cell, as observed by Miroslavov and Kravkina (1991) in mountain plants grown at high elevation, potentially enhancing energy production under low O_2 conditions.

The relative impact of the low partial pressures of CO_2 and O_2 on plant performance may differ. Based on our results, the effects of O_2 deficiency under reduced air pressure conditions could be the primary drivers of the physiological responses of our targeted species. Previous studies (Daunicht and Brinkjans, 1992; Ferl et al., 2002) have suggested that hypoxia is the major stress factor under reduced air pressure conditions, although hypobaria should not be simplistically equated with hypoxia (Paul et al., 2004, Zhou et al 2017). Furthermore, some studies have shown that some of the stress responses associated with reduced air pressures can be ameliorated by increasing the ppO_2 (e.g. Paul et al., 2004; He et al., 2007; He et al., 2013; Paul et al., 2017; Zhou et al., 2017).

On the other hand, the decrease in $\rho\rho O_2$, which leads to a lower photorespiration rate can partially compensate for the aforementioned effect of the decrease in $\rho\rho CO_2$. However, the decrease in O_2 availability could not be compensated for, and plants should activate alternative metabolic pathways for ATP production. For instance, Abbas et al. (2022) identified a genetic mechanism that alters the sensitivity of the oxygen-sensing system in plants at high elevations. Under our experimental conditions, C is likely not a limiting factor to plant growth at reduced air pressure, given that NSCs are accumulated in the two species studied at 62 kPa.

3.5 Conclusions

This study highlights the significant impact of reduced air pressure on plant physiology, particularly in mountain ecosystems. Our results indicate that reduced air pressure can affect key physiological processes, including photosynthesis and growth rate with responses varying between species. Although reduced air pressure reduces the availability of CO_2 and O_2, our finding suggests that oxygen deficiency plays a more dominant role than carbon limitation in shaping plant performance. Alpine species appear to cope with these conditions through strategies such as slower growth and enhanced carbohydrate storage. These results highlight the importance of considering reduced air pressure as a critical factor in understanding plant responses to high-elevation environments and climate change. Further research is needed to disentangle the complex interactions between

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reduced air pressure, CO_2 and O_2 availability, and other environmental factors to better predict the impacts on plant adaptation in alpine ecosystems and future ecosystem composition.

4. Materials and Methods

4.1. Plant material and experimental design

The two plant species chosen for the study, *Hieracium pilosella* L. and *Trifolium pratense* L., belong to the Asteraceae and Fabaceae families, respectively. *Trifolium pratense*, commonly known as red clover, is an herbaceous legume that is widely studied due to its agricultural importance, ecological significance, and medicinal properties. As a short-lived perennial species characterized by a deep taproot, it provides a good soil structuring effect. *Hieracium pilosella*, a forb, is a hairy perennial plant that favors dry areas and grows well on sandy and less fertile soils covering a broad elevational range (FloraVeg, 2024). This experiment was carried out in climatic chambers at the *terraXcube* Ecotron (https://terraxcube.eurac.edu/). The innovative aspect of the facility is its ability to reproduce characteristic alpine climate conditions, such as very low temperatures, high radiation, and reduced air pressure. Plants were placed in three Ecotron chambers of 27 m^3, where the air pressure was maintained at 85, 75, and 62 kPa corresponding to elevations of 1500, 2500, and 4000 m above sea level (a.s.l.), respectively. Data loggers were installed in the chambers to monitor key physical parameters: air temperature, relative humidity (RH), photosynthetically active radiation (PAR), air pressure, and the spectral characteristics of the LED lighting system (Supplementary Fig. S2, S3, S4, S5 and S6). Diurnal variations in environmental parameters were set according to the field conditions measured by a climate station located at 1500 m a.s.l. in the long-term Socio-Ecological Research (LTSER) site in the Matsch Valley (South Tyrol, Italy; 46°41'04.2"N, 10°35'08.5"E). Temperature and relative humidity were maintained consistently across all three chambers, with temperature ranging from 12°C to 24°C and relative humidity from 30% to 60%.

In May 2023, 30 healthy individuals of the two targeted species, all at a comparable early phenological stage, with unfolded leaves but no visible inflorescencewere collected by extracting soil–plant plugs from sites at 1500 m a.s.l. Bulk soil (Ah horizon) collected from the site was sieved to < 4 mm. Each plug was then transplanted into a 1-L pot (1 plug per pot) filled with bulk soil and randomly distributed across the 3 chambers, with 20 pots per chamber (10 for *H. pilosella* and 10 for

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T. pratense). An artificial irrigation system supplied UV-sterilized water at a rate of 33 mL per pot per every other day (2 L h^{-1} flow rate, equivalent to 1 min of watering every two days).

4.2. Growth parameter determination

The following growth parameters were determined according to standard protocols (Pérez-Harguindeguy et al., 2013): shoot and root biomass, leaf dry mass content (LDMC), specific leaf area (SLA), relative growth rate (RGR), and root to shoot biomass ratio (Root:Shoot). Then, nine and two fully expanded leaves were randomly sampled from *T. pratense* and *H. pilosella* plants, respectively, scanned, and weighed (mg) to obtain the fresh weight. They were then dried for 72 h at 70°C and reweighed (mg) to obtain the dry weight. The leaf area (cm^2) was estimated using an Epson GT5000 and processed using an image analyzer (ImageJ). At the end of the experiment, the aboveground and underground biomass was collected and oven-dried to estimate the plant dry weight biomass. The dry biomass was then used to determine the total nitrogen (N) and carbon (C) contents and C stable isotopes (see below).

4.3. Gas exchange measurements

Measurements of leaf gas exchange were carried out on one expanded leaf per plant around midday (11:00–15:00 h). Leaf net photosynthesis (A), stomatal conductance (g_s), and transpiration (E) were measured using a portable infrared gas analyzer (GFS-3000, Heinz Walz GmbH, Munich, Germany) with ambient barometric pressure values ranging from 62 to 110 kPa. The instrument was connected to a standard measuring head (3010S, maximum enclosed leaf area 1.25 cm^2) including a micro-quantum sensor to monitor the photosynthetically active radiation (PAR) and a thermocouple to measure temperature at the lower leaf surface. Conditions in the leaf cuvette were set to a photosynthetic photon flux density (PPFD) of 1300 µmol m^{-2} s^{-1}, a CO_2 mole fraction of 420 µmol mol^{-1}, an airflow rate of 300 µmol s^{-1} and a relative humidity (RH) of 50%. For measurement, a leaf was clamped in the chamber and exposed to a saturated light intensity until the assimilation rate reached a steady state.

To determine the carboxylation efficiency of Rubisco and the maximal photosynthetic rate, we determined the photosynthetic response to intercellular CO_2 (A-C_i). A-C_i curves were obtained by supplying the leaf chamber with increasing CO_2 concentrations at a constant saturating PPFD (1300 µmol m^{-2} s^{-1}), temperature (20°C), and RH (50%). The following CO_2 concentrations were applied:

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200, 400, 600, 800, 1000, 1200, and 1400 ppm. As CO_2 response curves are very laborious and time-consuming, we only determined the A-C_i response curves of the leaves of *T. pratense* and compared them between 85 and 62 kPa.

4.4. Chlorophyll fluorescence measurements

Chlorophyll fluorescence measurement is a non-invasive technique used to study the photosynthetic response in the first stages of damage caused by stress. Chlorophyll fluorescence of photosystem II (PSII) increases when excitation energy is not efficiently used by the photosynthetic apparatus. Measurements of chlorophyll fluorescence were performed around midday at the end of the experiment using a portable fluorimeter (Handy-PEA, Hansatech Institute Ltd, Norfolk, UK). After clamping the leaf-clip holder onto the leaf, the maximum fluorescence yield was measured by exposing the leaf to a saturating flash of 3500 µmol m^{-2} s^{-1} during exposure to natural illumination, and the photochemical efficiency of PSII (ϕPSII) was recorded as $\Delta F / F'_m = (F'_m - F) / F'_m$

After these measurements, the potential photochemical efficiency of PSII (F_v/F_m) was determined on dark-adapted leaves (over 20 min). F_v/F_m was calculated as follows: $F_v/F_m = (F_m - F_0) / F_m$ where F_0, F_v, and F_m are the initial, variable, and maximum fluorescence, respectively (Maxwell and Johnson, 2000). The non-photochemical quenching coefficient (*NPQ*, equivalent to $(F_m - F'_m) / F'_m)$) was calculated according to Oxborough et al. (1997).

4.5. Nitrogen, carbon, and chlorophyll content determination

The relative amount of chlorophyll was determined using a CCM-300 chlorophyll content meter (Opti-Sciences, Hudson, NH, USA) by measuring the absorbance of the leaves in the blue (400–500 nm) and red regions (600–700 nm). N and C contents were determined on approximately 2 mg of dry biomass, following the Dumas combustion method, using an elemental analyzer (Flash 2000, Thermo Scientific). The C:N ratio was then determined.

4.6. Non-structural carbohydrate determination

The non-structural carbohydrate (NSC) content of the dry biomass (leaves and roots) was extracted in boiling water for 5 min. The NSC content of leaves and roots was obtained by soaking approximately 30 mg of plant extracts in a water bath with 0.5% Clarase 900 in 0.1 M acetate buffer

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(pH 4.6) at 37°C for 48 h. Clarase 900 is a mixture of several digestive enzymes that hydrolyze starch and sucrose to hexoses. After converting fructose to glucose with P-glucose-isomerase, free glucose plus fructose was determined by spectrophotometry using a glucose-specific assay (Azcón-Bieto and Osmond, 1983).

4.7. Carbon isotope discrimination ($\Delta^{13}C$)

Carbon isotope discrimination ($\Delta^{13}C$) is a non-destructive, time-integrated indicator of plant physiological responses that does not require continuous monitoring. It is closely linked to the ratio of intercellular to ambient CO_2 concentration (Ci/Ca), which reflects both stomatal conductance and photosynthetic activity. Under hypobaric conditions, $\Delta^{13}C$ is particularly informative for two main reasons: First, reduced atmospheric pressure is typically associated with lower air water content, which can increase transpiration and lead to water stress. In this context, $\Delta^{13}C$ serves as a useful proxy for assessing how plants regulate stomatal behavior and optimize water-use efficiency. Second, $\Delta^{13}C$ provides insight into Rubisco activity, as hypobaric environments alter the CO_2/O_2 partial pressure ratio, thereby influencing the balance between carboxylation and oxygenation. Because Rubisco discriminates more strongly against ^{13}C (~27‰) than stomata (~ 4‰), a decrease in ^{13}C discrimination, reflected by greater incorporation of ^{13}C into plant tissue could indicate enhanced carboxylation efficiency and reduced limitations on CO_2 assimilation.

The oven-dried leaves and roots were ground into fine powder for the analysis of the carbon isotopic discrimination ($\Delta^{13}C$). Approximately 0.3 mg of the samples was weighed into a tin cup and combusted in an elemental analyzer (Flash 2000, Thermo Scientific) coupled to an isotope ratio mass spectrometer (Delta V Advantage, Thermo Scientific) by a Conflo III interface. Values were expressed as the $^{13}C/^{12}C$ ratio relative to the Pee Dee Belemnite standard.

4.8. Statistical analysis

Data analysis was performed using R (v. 4.3.0; R Core Team, 2022). The main effects of air pressure (85, 75, and 62 kPa), plant organ (roots *vs*. leaves), and their interaction were tested using plants as replicates. The effects of air pressure and organ type on carbon isotope discrimination and non-structural carbohydrates concentrations were assessed with two-way ANOVA. One-way ANOVA was carried out to test the effect of air pressure on gas exchange parameters, chlorophyll fluorescence, chlorophyll content, and biomass. Differences in carbon isotope discrimination between *H. pilosella* and *T. pratense* were also tested with a

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one-way ANOVA. Where ANOVA indicated significant effects, Tukey's Honest Significant Differences (HSD) test was applied for post-hoc comparisons. Statistical significance was set at $p < 0.05$. To examine the effects of air pressure on the A-Ci curves, analysis of covariance (ANCOVA) was performed with models including Ci and air pressure terms (different intercepts, the same slope). Graphs were generated using the R 'ggplot2' package (Wickham, 2016). The number of replicates is reported in the table and figure legends. When necessary, variables were log-transformed prior to analysis to satisfy the assumption of homoscedasticity.

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Authors' contributions

M.D., G.N., P.I., and N.P. conceived the idea of the study; all co-authors conducted the Ecotron experiment; B.E. and S.L. performed the measurements; B.E. analyzed the data; B.E. wrote the first draft of the manuscript, and all co-authors contributed to the final version of the manuscript.

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Conflicts of interest: Authors have no conflicts of interest to declare.

Data availability statement

Data is available on Zenodo (10.5281/zenodo.17531980)

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Table 1. Statistical analysis of the effect of air pressure on photosynthesis (A), stomatal conductance (gs), intercellular to ambient CO_2 ratio (C_i:C_a), plant dry weight (Plant DW), root dry weight (Root DW), shoot dry weight (Shoot DW), root to shoot ratio (R/S ratio), relative growth rate (RGR), chlorophyll content (Chl. Content), and photochemical efficiency of PSII (ϕPSII) in *Hieracium pilosella* and *Trifolium pratense*. Significant effects were assessed with one-way ANOVA conducted across all air pressure treatments *F*-values are shown. Levels of statistical significance are indicated with asterisks (***$p < 0.001$; **$p < 0.005$; *$p < 0.05$; ns, not significant) ($n = 7$).

	Species			
	H. pilosella		*T. pratense*	
Plant trait	*F*	*p*	*F*	*p*
A	6.336	**		ns
g_s	4.87	*		ns
C_i:C_a ratio	3.786	*	4.978	*
Plant DW	5.768	*	6.642	**
Root DW	5.059	*	6.21	*
Shoot DW	4.293	*		ns
R/S ratio		ns	5.787	*
RGR	4.419	*	12.35	***
Chl. Content	84.72	***	48.44	***
ϕPSII	3.605	*		ns

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Table 2. Mean values (± standard deviation) of relative growth rate (RGR), specific leaf area (SLA), leaf dry matter content (LDMC), leaf N concentration (Leaf N), leaf carbon to nitrogen ratio (Leaf C:N ratio), chlorophyll content (SPAD units), fraction of energy dissipated as heat (non-photochemical quenching, *NPQ*), the photochemical efficiency of the PSII (ϕPSII), and potential efficiency of PSII (F_v/F_m) in *Hieracium pilosella* grown under 85, 75, or 62 kPa (n = 7).

	Air pressure (kPa)		
Plant traits	**85**	**75**	**62**
RGR ($g \cdot g^{-1} \cdot week^{-1}$)	0.33 ± 0.08 (a)	0.20 ± 0.09 (ab)	0.18 ± 0.10 (b)
SLA ($cm^2 \cdot g^{-1}$)	144.7 ± 27.7 (a)	121.2 ± 25.5 (a)	124.5 ± 11.9 (a)
LDMC ($mg \cdot g^{-1}$)	210.3 ± 37.3 (a)	182.5 ± 29.4 (a)	202.2 ± 36.8 (a)
Leaf N ($mg \cdot cm^{-2}$)	0.92 ± 0.5 (a)	0.29 ± 0.1 (b)	0.65 ± 0.3 (ab)
Leaf C/N ratio	37.7 ± 12.6 (a)	32.2 ± 8.4 (a)	33.4 ± 10.9 (a)
SPAD units	57.4 ± 6.2 (a)	59.0 ± 5.2 (a)	31.4 ± 4.3 (b)
NPQ	0.35 ± 0.29 (a)	0.14 ± 0.11 (a)	0.3 ± 0.2 (a)
ϕPSII	0.756±0.02 (ab)	0.758 ± 0.02 (a)	0.731 ± 0.03 (b)
(F_v/F_m)	0.77 ± 0.1 (a)	0.82 ± 0.01 (a)	0.78 ± 0.08 (a)

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Table 3. Mean values (± standard deviation) of relative growth rate (RGR), specific leaf area (SLA), leaf dry matter content (LDMC), leaf N concentration (Leaf N), leaf carbon to nitrogen ratio (Leaf C:N ratio), chlorophyll content (SPAD units), the fraction of energy dissipated as heat (non-photochemical quenching, *NPQ*), the photochemical efficiency of PSII (ϕPSII), and the potential efficiency of PSII (F_v/F_m) in *Trifolium pratense* grown under 85, 75, or 62 kPa (n = 7).

	Air pressure (kPa)		
Plant traits	**85**	**75**	**62**
RGR ($g \cdot g^{-1} \cdot week^{-1}$)	0.47 ± 0.06 (a)	0.47 ± 0.13 (a)	0.23 ± 0.08 (b)
SLA ($cm^2 \cdot g^{-1}$)	225.6 ± 26.1 (a)	244.3 ± 65.3 (a)	191.0 ± 24.6 (a)
LDMC ($mg \cdot g^{-1}$)	250.2 ± 44.4 (a)	257.6 ± 22.1 (a)	281.0 ± 30.1 (a)
Leaf N ($mg \cdot cm^{-2}$)	2.57 ± 1.1 (a)	3.57 ± 1.5 (a)	2.67 ± 0.84 (a)
Leaf C/N ratio	21.9 ± 4.7 (a)	21.6 ± 4.4 (a)	18.1 ± 2.4 (a)
SPAD units	43.1 ± 2.7 (a)	44.9 ± 3.7 (a)	32.8 ± 2.4 (b)
NPQ	0.17 ± 0.08 (a)	0.23 ± 0.11 (a)	0.17 ± 0.07 (a)
ϕPSII	0.77 ± 0.02 (a)	0.75 ± 0.08 (a)	0.76 ± 0.02 (a)
F_v/F_m	0.79 ± 0.01 (a)	0.77 ± 0.13 (a)	0.80 ± 0.02 (a)

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Table 4. Effects of air pressure (P), plant organ (Org), and their interaction on the non-structural carbohydrate (NSC) content, carbon isotope discrimination ($\Delta^{13}C$), carbon to nitrogen ratio (C:N ratio), and leaf nitrogen content (N) in *Hieracium pilosella* and *Trifolium pratense*. Significant effects were assessed using ANOVA across all air pressure treatments. *F* and *p* values are shown; ns, not significant ($n = 7$).

		NSC		$\Delta^{13}C$		C:N ratio		N	
		F	*p*	*F*	*p*	*F*	*p*	*F*	*p*
T. pratense	P	7.03	0.009	12.18	<0.001		ns		ns
	Org	35.43	<0.001	13.16	<0.001		ns	6.8	0.013
	P×Org	19.03	<0.001			5.351	0.009		ns
H. pilosella	P		ns	11.56	<0.001				
	Org	15.54	<0.001	11.90	0.0014				
	P×Org	10.06	<0.001						

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Figure legends

Figure 1. Effect of air pressure [kPa] on the root and shoot dry weight [g] in *Hieracium pilosella* and *Trifolium pratense*. Shoots are represented by green bars (upper part) and roots are represented by brown bars (lower part). Different letters indicate significant differences in the total plant biomass (Root + Shoot) among the three air pressures treatments (85, 75 and 62 kPa) according to Tukey's HSD test ($p < 0.05$). Data are presented as geometric means (bars) with standard deviation (SD) indicated by vertical lines ($n = 7$).

Figure 2. Effect of air pressure [kPa] on the Root:Shoot biomass ratio in *Hieracium pilosella* and *Trifolium pratense*. Different letters indicate significant differences among the three air pressure treatments (85, 75 and 62 kPa) according to Tukey's HSD test ($p < 0.05$). Data are presented as geometric means (bars) with the standard deviation (SD) indicated by vertical lines (n = 7).

Figure 3. Effect of air pressure [kPa] on photosynthesis and stomatal conductance (gs) in leaves of *Hieracium pilosella* and *Trifolium pratense*. Different letters indicate significant differences among the three air pressure treatments (85, 75 and 62 kPa) according to Tukey's HSD test ($p < 0.05$). Data are presented as geometric means (bars) with the standard deviation (SD) indicated by vertical lines (n = 7).

Figure 4. Effect of air pressure [kPa] on the ratio of the intercellular to the ambient partial pressure of log CO_2 (C_i:C_a ratio) in the leaves of *Hieracium pilosella* and *Trifolium pratense*. Different letters indicate significant differences among the three air pressure treatments tested in the chambers (85, 75 and 62 kPa) according to Tukey's HSD test ($p < 0.05$). Data are presented as geometric means (bars) with the standard deviation (SD) indicated by vertical lines (n = 7).

Figure 5. Effect of air pressure [kPa] on the carbon isotope discrimination ($\Delta^{13}C$) in the leaves and roots of *Hieracium pilosella* and *Trifolium pratense*. Different letters indicate significant differences among the three air pressure treatments (85, 75 and 62 kPa) according to Tukey's HSD test ($p < 0.05$). Data are presented as geometric means (bars) with the standard deviation (SD) indicated by vertical lines (n = 7).

Figure 6. Effect of air pressure [kPa] on the non-structural carbohydrates (NSCs) in the leaves and roots of *Hieracium pilosella* and *Trifolium pratense*. Different letters indicate significant differences among the three air pressure treatments (85, 75 and 62 kPa) according to Tukey's HSD test ($p < 0.05$). Data are presented as geometric means (bars) with the standard deviation (SD) indicated by vertical lines (n = 7).

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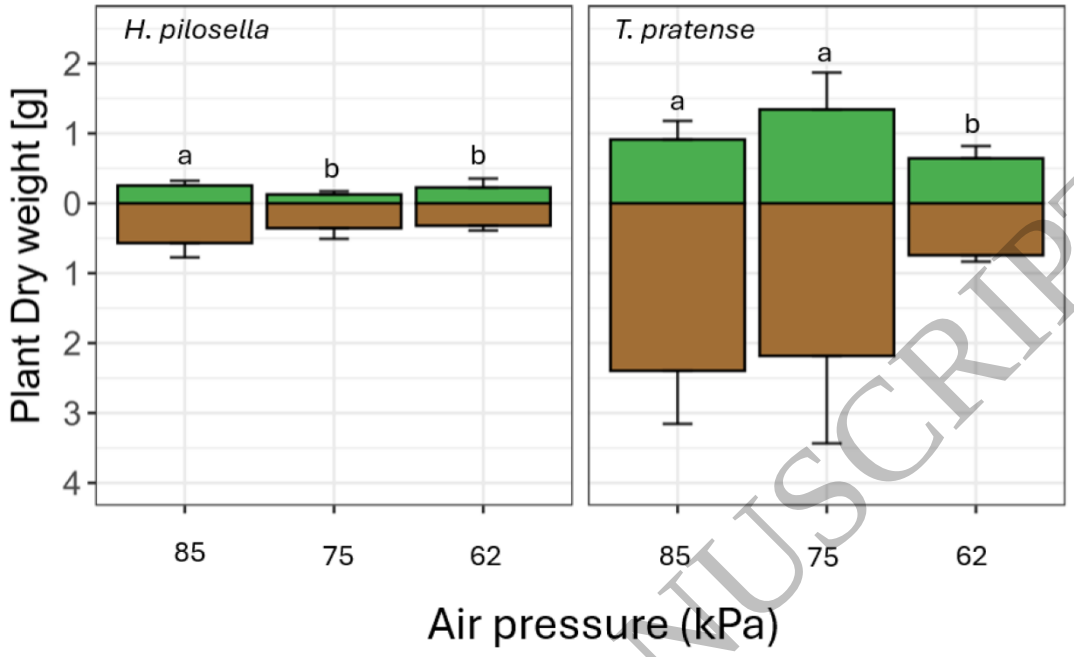

Fig. 1.

Figure 1
165x113 mm (x DPI)

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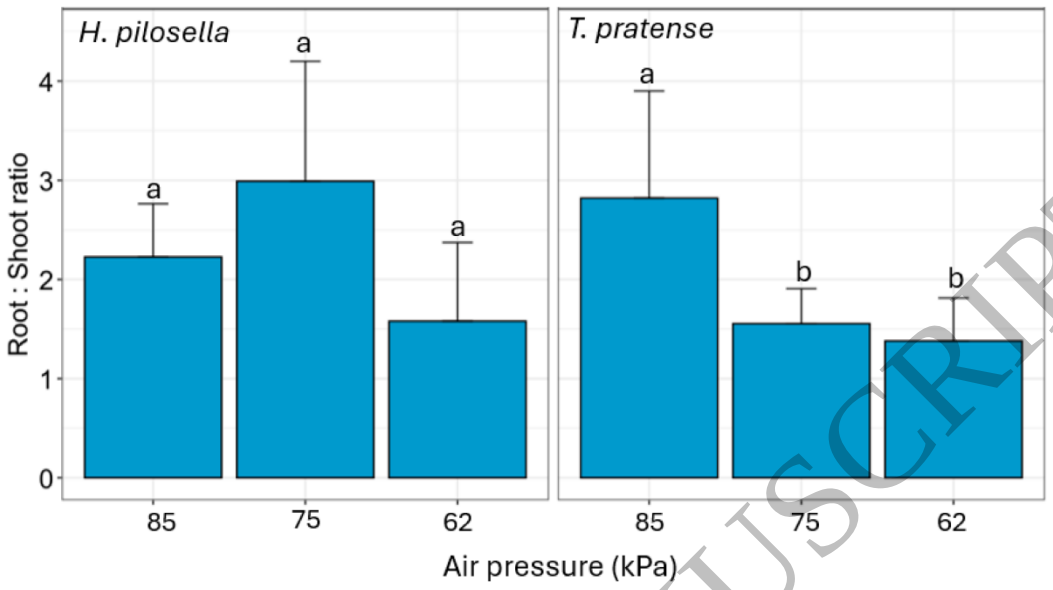

Fig. 2.

Figure 2
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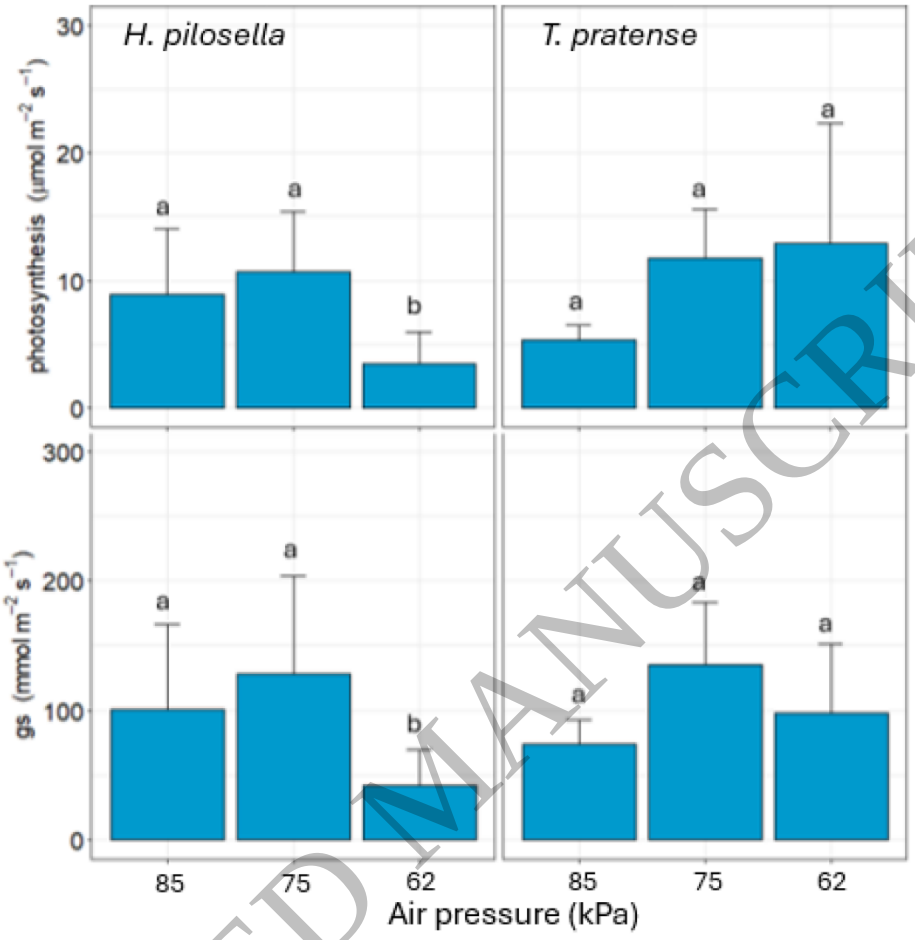

Fig. 3.

Figure 3
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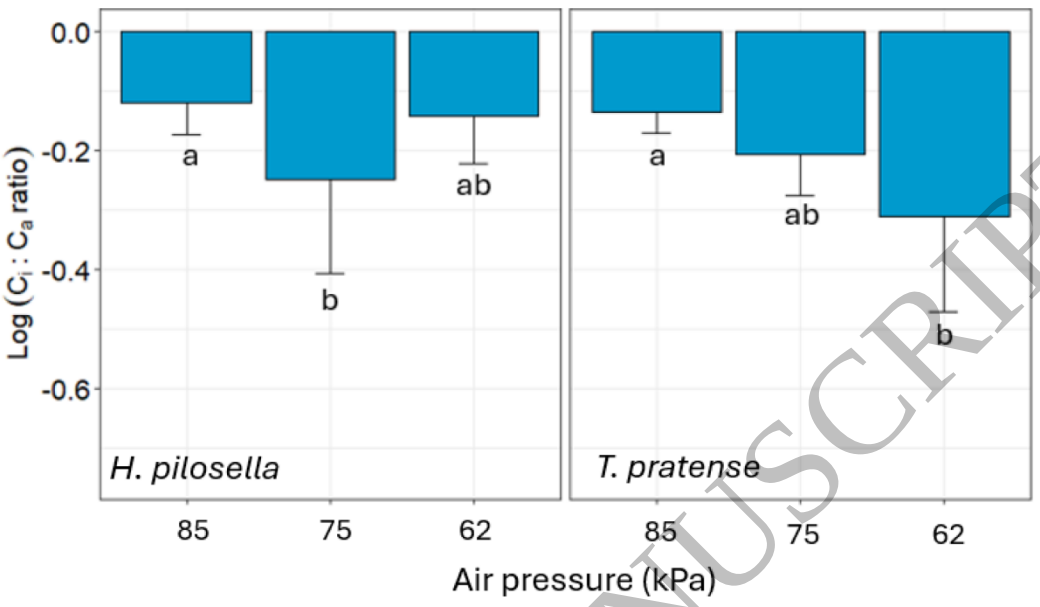

Fig. 4.

Figure 4
165x102 mm (x DPI)

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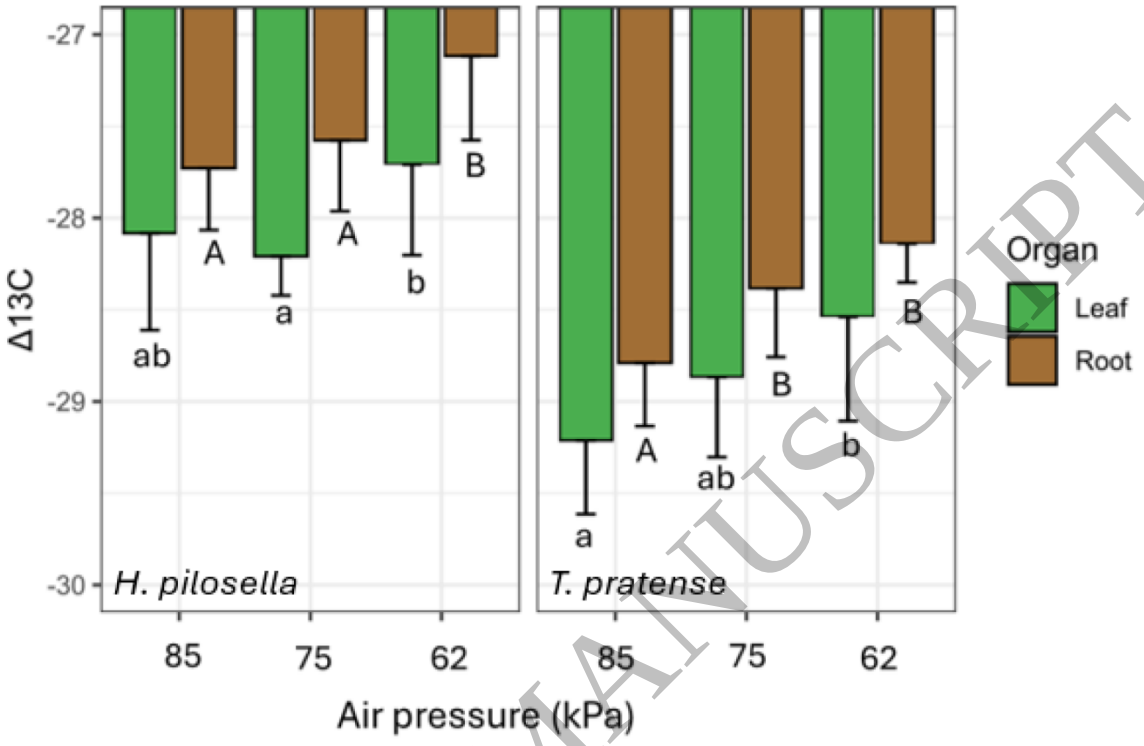

Fig. 5.

Figure 5
165x122 mm (x DPI)

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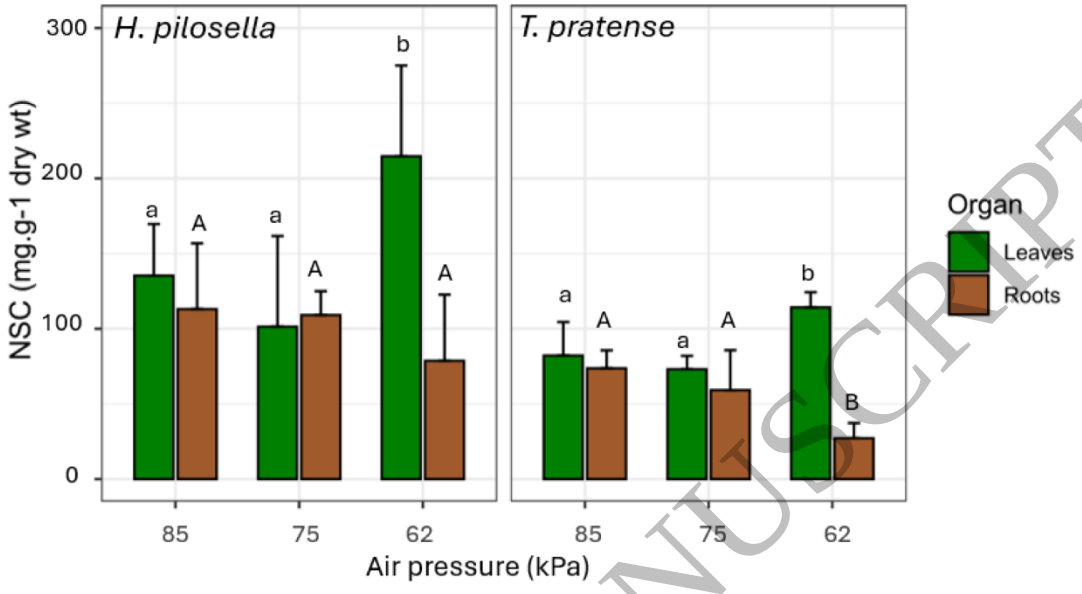

Fig. 6.

Figure 6
165x101 mm (x DPI)

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