

**MORPHOLOGICAL AND PHYSIOLOGICAL CHANGES IN GRASSES AND  
HERBACEOUS SPECIES UNDER THE COMBINED EFFECT OF RISING  
ATMOSPHERIC CO<sub>2</sub> CONCENTRATION AND LIMITED PHOSPHORUS  
FERTILIZATION**

*A controlled growth chamber experiment on *Holcus lanatus*, *Solanum dulcamara*  
and *Panicum Miliaceum**



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## Preface

This thesis was written as part of the MSc. in Environmental Sciences, program Sustainable Development - Global Change and Ecosystems, at Utrecht University. Use was made of the facilities and machines of Utrecht University in collaboration with the Biology Department, TNO and Utrecht Botanical Gardens. I was supervised by Dr. Hugo de Boer who assisted me during the whole process of the thesis. I would like to thank him for his advice, feedback and providing me with the plant seeds and materials making it possible to perform a good experiment. I would also to thank Shuqiong Wang for helping me with the calculation of nutrient concentration and creation of the nutrient solutions, Ineke Roeling and Rémon Saaltink for their help with using the different machines, Charlotte Ballard for proofreading and finally Dr. Jerry van Dijk as second reader for his feedback on the proposal.

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## Summary

Atmospheric CO<sub>2</sub> concentration ( $C_a$ ) has been continuously rising due to direct and indirect anthropogenic activities since the industrial revolution. Thanks to this  $C_a$  rise, photosynthetic rate and net primary productivity (NPP) of plants is increasing. Subsequent ecophysiological changes results in altered biomass allocation and in changes in leaves area ( $A_{\text{leaf}}$ ) in relation to biomass that in turn can regulate plants growth. Furthermore, changes in the leaf nitrogen ( $N_{\text{leaf}}$ ) and leaf phosphorus ( $P_{\text{leaf}}$ ) content can affect the maximal RuBisCO limited rate of photosynthesis ( $V_{\text{cmax}}$ ) and maximum electron transport ( $J_{\text{max}}$ ), respectively. However, few studies have examined the role of nutrient limitation, especially phosphorus (P), in downregulating plant growth to rising  $C_a$ , despite its potential influence on the global carbon cycle. It is likely that fully coupled climate-carbon cycle model projections misrepresent future plant carbon sequestration since they do not integrate low soil phosphorus concentration ( $P_s$ ) even though soil P is particularly scarce in many ecosystems and expected to decrease in the future. The present study investigated the combined effect of rising  $C_a$  and low  $P_s$  on the total plant biomass ( $B_t$ ) and on its allocation to the roots or above ground section, especially to the canopy, on  $A_{\text{leaf}}$  in relation to  $B_t$  and leaves biomass ( $B_{\text{leaf}}$ ) and on  $N_{\text{leaf}}$  and  $P_{\text{leaf}}$  and on their relationships with  $V_{\text{cmax}}$  and  $J_{\text{max}}$ . Three species, *Holcus lanatus*, *Solanum dulcamara* and *Panicum mileaceum* were grown in three phytotrons with 150, 450 and 800 ppm respectively and treated with two nutrient solutions in which P was varied in relation to N (1N:1P, 1N:45P). Results suggest that low  $P_s$  could hamper NPP at the end of the century more strongly than at the present. Moreover, above-below biomass ratio ( $B_a:B_b$ ), leaf mass ratio (LMR), specific leaf area (SLA) and leaf area ratio (LAR) responses showed high variability between the three species, suggesting that plants can adopt very different strategies under the independent and interactive effect of rising  $C_a$  and low  $P_s$  thanks to their different species physiological characteristics and ontogeny. In addition, the low statistical significance of SLA and LAR responses could suggest that, if well lighted, plants no longer need to invest in  $A_{\text{leaf}}$  regardless of variations in  $C_a$  and in  $P_s$ . Finally,  $N_{\text{leaf}}$  and  $P_{\text{leaf}}$  decreased similarly with increasing  $C_a$ , independently from the P treatment, and further decreased under low  $P_s$ . Results of the interactive effect of rising  $C_a$  and  $P_s$  indicate that plants under low  $P_s$  could suffer a milder reduction in  $P_{\text{leaf}}$  at present  $C_a$  compared with glacial  $C_a$ . Moreover,  $N_{\text{leaf}}$  and  $P_{\text{leaf}}$  showed a positive but not very strong linear relationship with  $V_{\text{cmax}}$  and  $J_{\text{max}}$ , respectively. This confirms that  $N_{\text{leaf}}$  and  $P_{\text{leaf}}$  concentrations may be able to partially regulate the allocation of N to the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and of P to the electron transport chain (ETC), respectively. However, low  $P_s$  weakened these positive relationships.

## List of abbreviations often used in the text

$A_{\text{leaf}}$  = leaves area.

$B_a:B_b$  = above-below ground plant biomass.

$B_{\text{leaf}}$  = leaves biomass.

$B_t$  = total biomass.

$C_a$  = atmospheric  $\text{CO}_2$  concentration.

ETC = electron transport chain.

$J_{\text{max}}$  = maximum electron transport.

LAR = leaf area ratio.

LMR = leaf mass ratio.

N = nitrogen.

$N_{\text{leaf}}$  = concentration of nitrogen in the leaves.

NPP = net primary productivity.

$N_s$  = soil nitrogen concentration.

P = phosphorus.

$P_{\text{leaf}}$  = concentration of phosphorus in the leaves.

$P_s$  = soil phosphorus concentration.

RuBisCO = ribulose-1,5-bisphosphate carboxylase/oxygenase.

SLA = specific leaf area.

$V_{\text{cmax}}$  = maximal RuBisCO-limited rate of photosynthesis.

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# 1. Introduction

## 1.1 Plant responses to CO<sub>2</sub> fertilization

Atmospheric CO<sub>2</sub> concentration ( $C_a$ ) has been rising since the industrial revolution because of emissions caused by human-related activities. More specifically, over the last decade  $C_a$  has been rising at the average rate of 2,2 ppm yr<sup>-1</sup>, and in May 2017 the estimated globally averaged  $C_a$  at the surface was 409 ppm (Team,2017). For much of the last million years  $C_a$  has oscillated between 172 and 300 ppm (Lüthi et al.,2008). The IPCC (Intergovernmental Panel on Climate Change) estimates that  $C_a$  will have risen to 421-936 ppm by the year 2100 based on four 'Representative Concentration Pathways' (RCPs) (Moss et al.,2010). This projected  $C_a$  increase may enhance the plant absorption rate of carbon, net primary productivity (NPP), especially in the tropics (Nemani et al.,2003), via a stimulating effect on the increasing photosynthesis rate (Ainsworth & Rogers,2007). For instance, the RCP 8.5 scenario projects an increase of NPP of around  $63 \pm 27\%$  by the end of the century (Wieder et al.,2015). By means of this effect, known as CO<sub>2</sub> fertilization, plants could act as a carbon sink. Various simulations of the CMIP5 (Coupled Model Intercomparison Project Phase 5) ensemble show how the biosphere can favourite the terrestrial C uptake (Ahlström et al.,2012).

Global estimates of the biosphere response to elevated  $C_a$  rely on a detailed understanding of plant ecophysiological processes. Crucially, an increase in total biomass ( $B_t$ ), a consequence of the NPP increase (Baker et al.,2004; Bellassen et al.,2011; Cannell et al.,1998; Cole et al.,2009; Graybill & Idso,1993; Lewis et al.,2009; Martínez-Vilalta et al.,2008; Voelker et al.,2006), is associated with plant physiological responses leading to reallocation of biomass and changes in the ratio between leaves area ( $A_{leaf}$ ) and biomass. Indeed, even if the allocation of biomass seems to be dependent on species physiological characteristics, ontogeny and on different environmental conditions experienced by the plant (Poorter & Nagel,2000), many studies suggest that with rising  $C_a$  more biomass is allocated to the roots compared with the above ground section of the plant (Ainsworth & Long,2005; Ceulemans & Mousseau,1994; Eamus & Jarvis,1989) and more specifically to the leaves, causing an above-below ground biomass ( $B_a:B_b$ ) and leaf mass ratio (LMR) decrease (Centritto & Jarvis,1999; Hättenschwiler & Körner,1997). Moreover,  $A_{leaf}$  seems to increase proportionately less in comparison to leaves biomass ( $B_{leaf}$ ) and  $B_t$ , leading to a decrease in specific leaf area (SLA) and leaf area ratio (LAR) (Bazzaz,1990; Centritto & Jarvis,1999; Hättenschwiler & Körner,1997; Hättenschwiler et al.,1997; Rogers et al.,1996; Tissue & Lewis,2010). Decreases in  $B_a:B_b$ , LMR, SLA and LAR in turn seem partially responsible for down regulation of photosynthetic carbon uptake (Callaway et al.,1994; Evans & Poorter,2001; Hättenschwiler et al.,1997). Finally, rising  $C_a$  reduces both the concentration of nitrogen in the leaves ( $N_{leaf}$ ) (Cotrufo et al.,1998; Tissue & Lewis,2010) and the concentration of phosphorus in the leaves ( $P_{leaf}$ ), with a stronger effect on  $P_{leaf}$  (Tissue & Lewis,2010). A reduction in these leaf nutrients ( $N_{leaf}$  and  $P_{leaf}$ ) can affect photosynthetic activity through biochemical down regulation of photosynthetic traits ( $V_{cmax}$  and  $J_{max}$ ) (Domingues et al.,2010; Walker et al.,2014;). Indeed,  $N_{leaf}$  seems to regulate the allocation of N to ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (Ainsworth and Rogers,2007; Griffin et al.,2000; Lewis et al.,2004), a nitrogen-rich carbon-fixing enzyme (ibid), while  $P_{leaf}$  is

involved with the allocation of P to the electron transport chain (ETC) (Domingues et al.,2010), where many P-rich molecules (ATP, NADP and sugar-phosphates from the Calvin cycle) are assembled (ibid). More specifically, leaf concentration of orthophosphate (Pi) affects ETC through a feedback mechanism (Woodrow & Berry,1988). However, down regulation of  $V_{cmax}$  and  $J_{max}$  with rising  $Ca$  is not connected with a reduction in net photosynthetic activity since there is an improvement in photosynthetic efficiency (Osborne et al.,1997; Rietveld,2016).

Studies have generally observed proportionately stronger plant responses in the transition from glacial (200 ppm) to modern  $Ca$  (350 ppm) than from modern to future (700 ppm)  $Ca$  (Baker et al.,1990; Dippery et al.,1995; Gill et al.,2002; Peñuelas & Matamala,1990; Ward et al.,1999). This pattern may reflect several factors, including the adaptation of plants to lower  $Ca$  than they currently experience (Körner,2006; Sage & Cowling,1999; Saxe et al.,1998), and that the relative effect of short-term increases in  $Ca$  on photosynthesis in plants is higher at low  $Ca$ , and subsequently declines when  $Ca$  rises above modern levels (Farquhar et al. 1980).

## 1.2 Phosphorus limitation in natural ecosystems

Concurrently to  $Ca$ , plants responses are also dependent to soil nutrients availability. A shortage of soil nutrients affects photosynthesis and the physiology of plants and it is a further factor to take into consideration when investigating future plants responses (De Graaff et al.,2006; Goll et al.,2012; Lewis et al.,2010; Lukac et al.,2010). Nitrogen (N) and phosphorus (P) are considered the most important nutrients for plants. On the one hand, N is the major component of chlorophyll, the pigment that allows plants to absorb energy from light. It is also a major component of amino acids, the building blocks of proteins. Furthermore, it is a significant component of nucleic acids such as DNA and enzymes (Mendelu,2017). On the other hand, P is a constituent of cell membrane, certain proteins, all nucleic acids and nucleotides, and is required for all phosphorylation reactions. It is also a fundamental component of DNA and it holds a “high energy bond” as a part of the chemical structures adenosine diphosphate (ADP) and adenosine triphosphate (ATP), being the source of energy that drives the multitude of chemical reactions within the plant (Anon,2017). Plants require P for plant growth and development. On average the atomic ratio of N:P in the soil is 13:1 (Cleveland & Liptzin,2007), since soil phosphorus concentration ( $P_s$ ) is generally low with respect to soil N concentration ( $N_s$ ). P is often suboptimal for plant growth, particularly in some natural ecosystems, because of its extreme immobility in the soil and because of its open-ended cycle that drives it toward depletion over time (Nord & Lynch,2009). Examples include ecosystems with “white sand” soils such as those present in French Guyana (Raaimakers et al.,1995), or temperate forests such as those in New Zealand (Richardson et al.,2004). Natural weathering events are the main cause of soil P loss in many ecosystems, for example the Mendocino marine terrace in California, the Cooloola dune sequence in Australia and the island archipelago of Hawaii (ibid). Even vast geographical regions like south-western Australia and the Cape region in South Africa suffer severe soil P impoverishment (Lambers et al.,2006). Furthermore, rising  $Ca$  increases the amount of P required to sustain the increased growth rates (Conroy et al.,1986; Lewis et al.,2010; Vance et al.,2003), although P mineralization rates are likely to remain low and not capable of satisfying this increased demand (Cleveland et al.,2013). Finally,



P will become increasingly less available also to crops, since global reserves of rock phosphate are depleted (Cordell et al.,2009; Lambers et al.,2006).

### 1.3 Plant responses to low $P_s$ and to its interactive effect with rising $Ca$

#### *Biomass growth and biomass allocation ( $B_t$ , $B_a:B_b$ , LMR)*

Low  $P_s$  results in numerous perturbations to plant growth and development and strongly lowers  $B_t$  (Conroy et al.,1988; Curtis & Wang,1998; Goudriaan & De Ruiters,1983; Moorby & Besford,1983; Zangerl & Bazzaz,1984). Despite this, the current literature does not clarify how this effect interacts with rising  $Ca$  (Wieder et al., 2015). Generally, plants suffering from a soil mineral shortage tend also to allocate a greater part of their biomass to the roots (Hermans et al.,2006). Low  $P_s$  results in a greater decrease in the biomass allocation to the above ground section of the plant than to the roots (Meharg et al.,1994; Hermans et al.,2006). Thus,  $B_a:B_b$ , already decreasing with rising  $Ca$ , is further reduced under low  $P_s$  (Eamus & Jarvis,1989; Ceulemans & Mousseau,1994). Similarly, LMR is also reduced, because of a greater decrease in the carbon allocation to the leaves than to the roots (Burslem & Turner,1996; Knox & Clarke,2005; Santiago et al.,2012). Generally, plants also seem to have a high capacity for adapting growth and biomass allocation patterns to soil type, P supply and neighbor presence (Sardans et al., 2004). Shifting biomass allocation to the roots might be a common strategy adopted by plants when the competitive pressure for nutrients increases (ibid). However, there are also plants that apply different strategies (ibid) because biomass allocation, as mentioned before, is dependent on many factors such as species physiological characteristics, ontogeny and other environmental growth conditions than only  $Ca$  and  $P_s$ . Finally, regarding the interactive effect of rising  $Ca$  and low  $P_s$  on biomass allocation, to the best of my knowledge, no literature exists.

#### *$A_{leaf}$ in relation to biomass (SLA, LAR)*

Low  $P_s$  lowers  $A_{leaf}$  relatively more than  $B_{leaf}$ , leading to a decreased SLA (Conroy et al.,1988; Radin & Eidenbock,1984; Tissue & Lewis,2010). However, low  $P_s$  seems to have a milder effect on SLA in high light conditions in comparison with low light conditions. Indeed, when light is not limited, plants do not have to invest in  $A_{leaf}$  (De Groot et al.,2001). Furthermore, the interaction of rising  $Ca$  and low  $P_s$  does not result in any statistically significant response of SLA (Tissue & Lewis,2010). By contrast, LAR seems affected by the interactive effect of rising  $Ca$  and low  $P_s$  (ibid). Indeed, LAR also decreases under low  $P_s$  because of a proportionately greater decrease in  $A_{leaf}$  than in  $B_t$  (ibid), and suffers a greater reduction in the transition from glacial to modern  $Ca$  than from modern to future  $Ca$  (ibid). However, as Feller et al. (2007) demonstrate, LAR under low  $P_s$  can also experience the opposite response. Generally, LAR can be partitioned into the product of LMR and SLA, and most of the variation in LAR can be explained by mixed effects from these two parameters (Huxman et al.,1998).

#### *Leaf nutrients and their relationships with photosynthetic traits ( $N_{leaf}$ , $P_{leaf}$ , $V_{cmax}$ , $J_{max}$ )*

Variation in  $N_{leaf}$  and  $P_{leaf}$ , as already mentioned, can result in major changes in plant photosynthetic activities through the regulation of  $V_{cmax}$  and  $J_{max}$ . This regulation rule is normally reflected in a positive relationship between  $N_{leaf}$  and  $V_{cmax}$  (Domingues et al.,2010; Walker et

al.,2014) and between  $P_{leaf}$  and  $J_{max}$  (Domingues et al.,2010). However, the positive relationship between  $N_{leaf}$  and  $V_{cmax}$  seems partially weakened under low  $P_s$ , since  $N_{leaf}$  is not affected by low  $P_s$  while  $V_{cmax}$  decreases under low  $P_s$  (Tissue & Lewis,2010). Differently, low  $P_s$  leads to a lower concentration of both  $P_{leaf}$ , and  $J_{max}$ , therefore not affecting the positive relationship and the downregulatory role of  $P_{leaf}$  on  $J_{max}$ . Under low  $P_s$  conditions, differently than with rising  $Ca$  the down regulation of  $V_{cmax}$  and  $J_{max}$  is related with a down regulation of the net photosynthetic rates (Rietveld,2016). Despite this, once again, to the best of my knowledge, no current literature addresses the interactive effect of rising  $Ca$  and low  $P_s$  on  $P_{leaf}$  and  $N_{leaf}$  and therefore on the relationships between leaf nutrients and photosynthetic traits.

#### 1.4 Gap in knowledge and relevance of the research

Accordingly to the aforementioned literature review, it is unlikely that P supply will meet the increased plant nutrients demand generated from projected NPP increases, since soil P is particularly scarce in many ecosystems and P mineralization rates are low. However, until now scientific research on plant responses to rising  $Ca$  in combination with limited soil nutrients availability has mainly focused on N (Ainsworth & Long,2005; Ainsworth & Rogers,2007; Lewis et al.,2004; Peterson et al.,1999) and to a lesser extent on P (Ainsworth & Rogers,2007; Campbell & Sage,2006). Therefore, there is lack of studies on the influence of low  $P_s$  on plant growth to rising  $Ca$ , despite its potential downregulatory effect. As such, most fully coupled climate-carbon cycle models could misrepresent future plant carbon sequestration (Wieder et al.,2015), since low  $P_s$  under future  $Ca$  could limit NPP and in turn affect the dynamics of the carbon cycle (ibid). At the same time, it could-trigger ecophysiological mechanisms responsible for morphological changes such as biomass reallocation to roots and reduction of  $A_{leaf}$  in relation to biomass. These ecophysiological mechanisms in turn may downregulate the  $CO_2$  fertilization effect. Moreover, resulting modifications in  $N_{leaf}$  and  $P_{leaf}$  concentrations could directly interfere with photosynthesis when regulating N allocation to RuBisCO and P allocation to ETC, respectively. Decreased  $N_{leaf}$  and  $P_{leaf}$  and the plausible subsequent reduced nutrient allocation causing down-regulation of  $V_{cmax}$  and  $J_{max}$  in plants grown under elevated  $Ca$  and low  $P_s$  could be further potential ecophysiological and biochemical mechanisms limiting the  $CO_2$  fertilization effect. However, low  $P_s$  seems to weaken the positive relationship between  $N_{leaf}$  and  $V_{cmax}$ , suggesting that other mechanisms should prevail in the down regulation of  $V_{cmax}$  under this condition. Thus, it is highly relevant to understand whether the relationships between leaf nutrients and photosynthetic traits are further affected by the combined effect of low  $P_s$  and rising  $Ca$ .

## 1.5 Research questions and aim of the research

- 1- How does  $B_t$  and biomass allocation to the above-below ground sections, and more specifically to the leaves respond to the combined effect of rising  $Ca$  and low  $P_s$ ?
- 2- How does  $A_{leaf}$  in relation to biomass respond to the combined effect of rising  $Ca$  and low  $P_s$ ?
- 3- How do  $N_{leaf}$  and  $P_{leaf}$  respond to the combined effect of rising  $Ca$  and low  $P_s$ ? And how is their change reflected in the responses of  $V_{cmax}$  and  $J_{max}$ , respectively?

Based on these research questions the present study aims to find out how two C3 plants, a grass species, *Holcus lanatus* (*H.lanatus*) and a semi-woody herbaceous species, *Solanum dulcamara* (*S.dulcamara*), and a C4 grass, *Panicum mileaceum* (*P.miliaceum*), respond to the combined effect of rising  $Ca$  and low  $P_s$ . Thus, not only  $Ca$  but also  $P_s$  is varied to evaluate the difference in plant responses under low  $P_s$  compared with high  $P_s$ . High  $P_s$  was preferred over normal  $P_s$  to better visualize the effect of low  $P_s$ . Moreover, it is suggested to the reader of this manuscript, before further continuing the reading, to grasp the meaning of “combined effect”, a pivotal step for understanding the aim of the present study. Indeed, a combined effect of  $Ca$  and  $P_s$  can be both the result of the sum of their independent effects or the result of their interaction.

The present study aims to investigate the responses of different plant traits. Firstly, the key aim of this research is to investigate NPP changes through  $B_t$  responses. Secondly, biomass allocation to the above-below ground section and to the canopy through  $B_a:B_b$  and LMR responses is studied. Thirdly, the changes of  $A_{leaf}$  in relation to changes in  $B_t$  and in  $B_{leaf}$  are investigated, through SLA and LAR responses. Finally, the responses of  $N_{leaf}$  and  $P_{leaf}$  are investigated and related to the responses of  $V_{cmax}$  and  $J_{max}$ , respectively, all along the  $Ca$ - $P_s$  multi-factor treatment. To better understand the combined effect of the external factors ( $Ca$  and  $P_s$ ) on these relationships, the effect on  $N_{leaf}$  is compared with the effect on  $V_{cmax}$  and the effect on  $P_{leaf}$  is compared with the effect on  $J_{max}$ .

## 1.6 Hypotheses

The present study hypothesizes that plants responses to rising  $Ca$  are generally stronger in the transition from glacial to modern  $Ca$  than from modern to future  $Ca$ . Glacial, modern and future  $Ca$  levels are comparable to the ones adopted in the present study but still slightly different. Moreover, the low  $P_s$  discussed in the literature review are most of the time slightly different between the different literature sources and as well from the one adopted in the present study. Therefore, the accuracy of the hypotheses is not very high.

Most of the following hypotheses are based on the independent effect of rising  $Ca$  and low  $P_s$  because almost no literature about their interaction effect could be retrieved.

#### *Combined effect of rising Ca and low P<sub>s</sub> on biomass growth and biomass allocation*

B<sub>t</sub> is expected to increase with rising Ca and to decrease under low P<sub>s</sub>. However, from the literature it is not clear how biomass reduction under low P<sub>s</sub> would vary at the different Ca. Therefore, expectations regarding the interactive effect of rising Ca and low P<sub>s</sub> cannot not be defined. A higher amount of biomass is expected to be allocated to the roots compared with the above ground section with rising Ca, while under low P<sub>s</sub> it is expected that less biomass is allocated to the above ground section. Therefore, the combined effect of low P<sub>s</sub> with rising Ca is expected to further reduce B<sub>a</sub>:B<sub>b</sub> and LMR. However, because the allocation of biomass is also dependent on various environmental growth conditions, not only Ca and P<sub>s</sub>, on the characteristic physiological mechanisms of species and ontogeny the power of these hypotheses is rather low. Finally, because no literature was found on the interactive effect of rising Ca and low P<sub>s</sub> on biomass allocation, no expected results can be posited.

#### *Combined effect of rising Ca and low P<sub>s</sub> on A<sub>leaf</sub> in relation to biomass*

Generally, SLA and LAR are expected to decrease with both rising Ca and low P<sub>s</sub>. Indeed, A<sub>leaf</sub> is expected to increase less compared with B<sub>leaf</sub> and B<sub>t</sub> with rising Ca, while it is expected to decrease more than B<sub>leaf</sub> and B<sub>t</sub> under low P<sub>s</sub>. Therefore, with rising Ca low P<sub>s</sub> is expected to further reduce SLA and LAR. Nevertheless, LAR can also show an opposite effect under low P<sub>s</sub> and therefore these hypotheses are not expected to always hold true. On the one hand, the interaction of the external factors is not expected to affect SLA. On the other hand, this interaction is expected to affect LAR, since the effect of low P<sub>s</sub> is expected to be stronger in the transition from glacial to modern Ca than from modern to future Ca (ibid). However, under high light conditions, as the ones adopted in the present study, the effect of low P<sub>s</sub> on SLA is expected to be mild. This mild effect of low P<sub>s</sub> on SLA is expected to be partially reflected on LAR, since this parameter is the product of LMR and SLA.

#### *Combined effect of rising Ca and low P<sub>s</sub> on leaf nutrients and on their relationships with photosynthetic traits*

It is expected that N<sub>leaf</sub> and P<sub>leaf</sub> are positively related to V<sub>cm<sub>ax</sub></sub> and J<sub>max</sub> respectively. However, low P<sub>s</sub> is expected to weaken the positive relationship between N<sub>leaf</sub> and V<sub>cm<sub>ax</sub></sub>, because the two traits are expected to respond differently under low P<sub>s</sub>. N<sub>leaf</sub>, P<sub>leaf</sub>, V<sub>cm<sub>ax</sub></sub> and J<sub>max</sub> are all expected to decrease with rising Ca. By contrast, N<sub>leaf</sub> is not expected to show a response under low P<sub>s</sub> while P<sub>leaf</sub>, V<sub>cm<sub>ax</sub></sub> and J<sub>max</sub> are all expected to further decrease. However, no literature was found regarding the interaction of rising Ca and low P<sub>s</sub> on N<sub>leaf</sub> and P<sub>leaf</sub> and so on the relationships between leaf nutrients and photosynthetic traits, thus no expectations can be posited.

## 2. Materials and Methods

### 2.1 Plant material

Three species were grown and treated under the same conditions with different levels of  $Ca$  and  $P_s$ . The chosen species were *S.dulcamara* (bittersweet), a semi-woody C3 herbaceous perennial vine, *H.lanatus* (Yorkshire fog), a perennial C3 grass and *P.miliaceum* (Proso millet), an annual C4 grass used as a crop. Seeds of each species were sown in Primasta® potting soil in their respective  $Ca$  growth chambers. Plants of the same species for each chamber were grown together in the potting soil in one bin. When the seedlings seemed robust enough to withstand transplantation (around six weeks), 24 individuals per species were selected from each bin and repotted to individual pots filled with crystal sand with the size of approximately one liter. Then, they were left one week in the sand to adapt at the new soil before to start the nutrient treatment. The sand had an average diameter of 2,5 mm and had been heated and filtered so as not to contain any seeds or microbes. Nutrients contamination of sand was prevented before the nutrients treatment started.

### 2.2 Treatment conditions

Three phytotrons with  $Ca$  swinging around low (150), ambient (450) and high (800) ppm respectively were used for growing the plants. The temperature was kept constant at 21 °C during the day, and was lowered to 17 °C during the night. Plants were organized in parallel lines over long tables and irradiated homogeneously at around one meter distance from 08:00 to 18:00 by high-intensity discharge lamps with sunlight-similar spectrum and irradiance of around 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Relative humidity (RH) was around 70 % during the light period and around 90 % during the dark period. Thijs Rietveld together with Shuqiong Wang prepared the nutrient solutions for *H.lanatus* and *S.dulcamara*. Rietveld also took care of these plants all along their growth and treatment. The same nutrient solution preparation and treatments procedures applied by Rietveld were adopted for *P.miliaceum*. Pots were watered every two days in the morning with either 0,3, 0,4 and 0,5 liters of water that corresponded to the three periods of nutrient solution treatment (week 1-5, 6-8 and 9-11 respectively). Plants were observed daily to ensure no stress had occurred. Plants were grown in the sand with a nutrient solution containing a ratio of N:P of either 1:1 (high P) or 45:1 (low P). Only P was varied, N was kept high and K and other micronutrients were not limited and stayed the same level throughout the experiment. In total 12 plants per species were grown at same  $Ca$  and under the same nutrient treatment. However, not all individuals could be selected for traits measurements at the end of the nutrient treatment. Plants received sufficient water fed from the bottom of the pots and were treated weekly with the nutrient solution created in the lab. So that the plants were able to adjust to the nutrient solution, the amount of nutrients was built up along the treatment. In the first three weeks plants received 15 ml of solution. The next two weeks this was increased to 50 ml solution. During the following three weeks the solution was twice as concentrated and again 50 ml per week was given to the plants. During the final three weeks the solution was three times as concentrated as the initial solution and again 50 ml was given weekly. The amount of nutrients and the composition of the solutions can be found in tables 1 and 2.

**Table 1. Chemicals used to create the two different nutrient solutions with high and low content of P (High P, Low P) given in the concentration of mass per liter and their composition for week 1-5, 6-8 and 9-11. The top four chemicals were used for the macronutrients N, P and K and the rest of the chemicals were used for the several micronutrients (adapted from Thijs,2016).**

Chemical	High P g/L			Low P g/L		
	Week 1-5	Week 6-8	Week 9-11	Week 1-5	Week 6-8	Week 9-11
KNO <sub>3</sub>	0,29	0,57	0,86	0,29	0,57	0,86
Ca(NO <sub>3</sub> ) <sub>2</sub>	0,51	1,02	1,52	0,51	1,02	1,52
KH <sub>2</sub> PO <sub>4</sub>	0,44	0,88	1,32	0,01	0,02	0,03
KCl	1,44	0,60	0,34	1,48	1,26	1,04
<b>Micronutrients (mg/L)</b>						
CaCl <sub>2</sub> . 2H <sub>2</sub> O	378,49	378,49	378,49	378,49	378,49	378,49
MgSO <sub>4</sub> . 7H <sub>2</sub> O	237,51	237,51	237,51	237,51	237,51	237,51
FeSO <sub>4</sub> .7H <sub>2</sub> O	40,40	40,40	40,40	40,40	40,40	40,40
EDTA-2Na.2H <sub>2</sub> O	54,09	54,09	54,09	54,09	54,09	54,09
CuSO <sub>4</sub> .5H <sub>2</sub> O	0,16	0,16	0,16	0,16	0,16	0,16
H <sub>3</sub> BO <sub>3</sub>	4,44	4,44	4,44	4,44	4,44	4,44
MnSO <sub>4</sub> .H <sub>2</sub> O	1,49	1,49	1,49	1,49	1,49	1,49
Na <sub>2</sub> MoO <sub>4</sub> .H <sub>2</sub> O	0,20	0,20	0,20	0,20	0,20	0,20
ZnSO <sub>4</sub> .7H <sub>2</sub> O	0,88	0,88	0,88	0,88	0,88	0,88

**Table 2. Concentrations of the macro and micronutrients derived from the chemicals used to create the two different nutrient solutions with high and low content of P (High P, Low P) of table 1 given in the concentration of mass per liter for the different periods of nutrient treatment (week 1-5, week 6-8, week 9-11) and for the total nutrient treatments period (total) (adapted from Thijs,2016).**

Nutrient	High P g/L (1:1)			Total (g)	Low P g/L (45:1)			Total (g)
	Week 1-5	Week 6-8	Week 9-11		Week 1-5	Week 6-8	Week 9-11	
N	0,10	0,20	0,30	0,60	0,10	0,20	0,30	0,60
P	0,10	0,20	0,30	0,60	0,002	0,004	0,007	0,013
K	0,99	0,79	0,89	2,67	0,89	0,89	0,89	2,67
<b>Micronutrients in mg/L</b>								
Ca	378,49	378,49	378,49	1135,47	378,49	378,49	378,49	1135,47
Mg	237,51	237,51	237,51	712,53	237,51	237,51	237,51	712,53
Fe	94,48	94,48	94,48	283,44	94,48	94,48	94,48	283,44
Cu	0,16	0,16	0,16	0,48	0,16	0,16	0,16	0,48
B	4,44	4,44	4,44	13,32	4,44	4,44	4,44	13,32
Mn	1,49	1,49	1,49	4,47	1,49	1,49	1,49	4,47
Mo	0,20	0,20	0,20	0,60	0,20	0,20	0,20	0,60
Zn	0,88	0,88	0,88	2,64	0,88	0,88	0,88	2,64

The multi-factor treatment of the present study resulted in the following six treatment combinations:

**Table 3.** The six treatment combinations adopted in the experiment. Three different Ca (150 ppm or low Ca, 450 ppm or ambient Ca, 800 ppm or high Ca) were intertwined with two different N:P solutions (N:P= 1 or low P, N:P= 45 or high P).

N:P treatments	Ca treatments		
	150 ppm	450 ppm	800 ppm
N:P= 1	Low Ca-low P	Ambient Ca-low P	High Ca-low P
N:P= 45	Low Ca-high P	Ambient Ca-high P	High Ca-high P

The two N:P solutions when injected into the sand resulted in two P<sub>s</sub>, low P<sub>s</sub> and high P<sub>s</sub>.

## 2.3 Plant functional traits measurements

### *Biomass and leaf traits*

Five months after sowing the seeds and 11 weeks since the nutrient treatment had begun the plants had grown enough for measurement. Stems were cut at their base, seeds (present only on *P.miliaceum*) were harvested and leaves were cut at their pistil basis. Simultaneously, pots with roots were refrigerated to block growth. Then leaves were photographed and their area was determined using ImageJ. Once A<sub>leaf</sub> was measured, roots were extracted from the sand. Thereafter stems, seeds, leaves and roots were dried at 70 °C for 48 hours. Finally, dried stems, seeds, leaves and roots were weighed by means of an analytical balance with digital precision scale. Weights of leaves, stems and seeds, when present, were summed to calculate B<sub>a</sub> and measurements of roots were adopted for B<sub>b</sub>. To calculate B<sub>t</sub>, B<sub>a</sub> and B<sub>b</sub> were summed:

$$B_t = B_a + B_b$$

To calculate B<sub>a</sub>:B<sub>b</sub>, B<sub>a</sub> was divided by B<sub>b</sub>:

$$B_a : B_b = \frac{B_a}{B_b}$$

To calculate LMR, B<sub>leaf</sub> was divided by B<sub>t</sub>:

$$LMR = \frac{B_{leaf}}{B_t}$$

To calculate SLA, A<sub>leaf</sub> was divided by B<sub>leaf</sub> of the same leaves:

$$SLA = \frac{A_{leaf}}{B_{leaf}}$$

Finally, to calculate LAR, LMR was multiplied by SLA:

$$LAR = LMR \times SLA$$

In total eight individuals per treatment combination were selected for all species to calculate  $B_t$ ,  $B_a$  and  $B_b$ . For  $A_{\text{leaf}}$  eight leaves per individual were selected in all the species. However, eight individuals per treatment combination were selected in *H.lanatus* and *S.dulcamara* while only six individuals could be selected in *P.miliaceum*.  $A_{\text{leaf}}$  in *H.lanatus* at low  $Ca$  and high  $P_s$  could not be measured.

### *Leaf nutrients*

To calculate  $N_{\text{leaf}}$  and  $P_{\text{leaf}}$ , dried leaves were first ground to a fine powder in a Retsch mm400 mixer mill. For  $N_{\text{leaf}}$  analysis samples between 1000 and 2000  $\mu\text{g}$  were weighed in tin containers of 8x5 mm using an analytical micro-balance and loaded in a 96-wells plate. Samples were then analyzed using a Carlo Erba NA1500 elemental analyzer (Thermo scientific) with the principle of "flash combustion". The corresponding order and weights of samples were entered in an excel sheet in a computer with dedicated EAGER300 software for further processing of the outputs of the machine. For  $P_{\text{leaf}}$ , samples between 20 and 35 mg were weighed and diluted in a small volumetric flask with 2,5 ml of 1 % Triton solution and 10  $\mu\text{g}$  of Selenium and Gallium. The final solutions were homogenized by means of a magnetic stirrer and 10  $\mu\text{g}$  of the solutions were pipetted to polished glass carrier disks. The disks with samples were then dried on a heating plate so that the liquid evaporated and the solid content was left. Finally, the disks with the solid samples were inserted in a S2 Picofox machine (Bruker) and analyzed using the principle of total reflection X-ray fluorescence spectroscopy (TXRF). The corresponding order, nutrients and weight characteristics of the sample solutions were entered in a computer with dedicated XRF Software Spectra7 for further processing of the outputs of the machine. To ensure reliability and improve the quality of the results many triplet samples were used. In total for both  $N_{\text{leaf}}$  and  $P_{\text{leaf}}$  five individuals per treatment combination were selected in all species.

### *Photosynthetic traits*

$V_{\text{cmax}}$  and  $J_{\text{max}}$  were calculated in *H.lanatus* and *S.dulcamara* in the course of the last week of nutrient treatment and the following week, just before the harvest of the plants. Measurements were conducted by Thijs Rietveld using a LI-COR LI6400XT. The block of the device was set at 21 °C, the light value was 1500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  to measure at saturated light, the reference  $Ca$  was 150/450/800 ppm depending on the  $Ca$  treatment and RH was kept around 70 %. The full area of the 6  $\text{cm}^2$  LI-COR cuvette was covered with healthy leaves.  $A/C_i$  curves was established with the  $Ca$  values 50, 100, 200, 300, 400, 600, 800, 1000, 1200, 1600, 2000 ppm. The data collected with the LI-COR were stored in excel files. With the data of the  $A/C_i$  curves,  $V_{\text{cmax}}$ ,  $J_{\text{max}}$  were determined using the program R.  $A/C_i$  curves were analyzed in R using the 'plantecophys' package (Duursma,2015). For *S.dulcamara* seven individuals in the high  $Ca$  phytotron under both the high and low P treatments were measured, while six individuals were measured for the other treatment combinations. Every day, the order of measuring individuals from different treatment combinations was mixed to prevent individuals from the same treatment combination being measured always the same hours, since the photosynthetic responses can vary during the day (Hastings et al., 1961). All measurements were completed between 10:00 and 15:00, since photosynthesis is usually more active in this part of the day (ibid).



## 2.4 Statistical analysis

Statistical analysis was performed in IBM SPSS Statistics 24.0, boxplots and scatter plots were created using Microsoft Excel 2016, while tables were created using Microsoft Word 2016. ANOVAs were run to check whether there was a statistically significant response of the different traits to the independent and interactive effect of the external factors. Species,  $P_s$  and  $Ca$  were set as independent variables while the different traits ( $B_t$ ,  $B_a:B_b$ , LMR, SLA, LAR,  $N_{leaf}$ ,  $P_{leaf}$ ,  $J_{max}$  and  $V_{cmax}$ ) were set as dependent variables. A first one-way ANOVA with species set as independent variable was run to investigate whether the dependent variables under the combined effect of rising  $Ca$  and low  $P_s$  responded differently between species. Since the dependent variables always responded differently between species a two-way ANOVA with  $Ca$  and  $P_s$  set as independent variables was run to check whether the dependent variables responded differently under the independent or interactive effect of changing  $Ca$  (150-450-800 ppm) and changing  $P_s$  (high-low) for each species. A second one-way ANOVA with  $Ca$  set as independent variable was run to check whether the dependent variables responded differently under the stepwise changes in  $Ca$  (150-450 ppm, 450-800 ppm) at a specific  $P_s$  (low, high) for each species. A third one-way ANOVA with  $P_s$  set as independent variable was run to check whether the dependent variables responded differently under the change in  $P_s$  (high-low) at a specific  $Ca$  (150,450,800 ppm) for each species. A first post-hoc Tukey HSD test with species set as independent variable was run to check whether the dependent variables responded differently between pairs of species. A second post-hoc Tukey HSD test with  $Ca$  set as independent variable was run to check whether the dependent variables responded differently under the stepwise changes in  $Ca$ , independently from changes in  $P_s$ , for each species.

Linear regressions were also performed to check the relationships of  $N_{leaf}$  (independent variable) with  $V_{cmax}$  (dependent variable) and of  $P_{leaf}$  (independent variable) with  $J_{max}$  (dependent variable). However, because  $V_{cmax}$  and  $J_{max}$  were derived from different leaves than  $N_{leaf}$  and  $P_{leaf}$ , linear regressions between individuals could not be performed. Although, an ecological linear regression between average of populations of the different treatment combinations could be run, for a total of six average values for each variable per species, one for each treatment combination. To get enough data for the linear regression, the values of different species were grouped together. Because  $V_{cmax}$  and  $J_{max}$  from *P.miliaceum* were not integrated in the study, only data of *H.lanatus* and *S.dulcamara* were grouped and a total of 12 average values for each variable was therefore obtained. An assumption of similar behavior between these two species was made even if they often showed statistically significant differences in their responses to the combination of rising  $Ca$  and low  $P_s$  (appendix A).

For all statistical tests the following scale for the significance levels was adopted: *ns* = not significant + =  $P \leq 0,10$  \* =  $P \leq 0,05$  \*\* =  $P \leq 0,01$  \*\*\* =  $P \leq 0,001$ . Test results were considered statistically significant if  $P \leq 0.05$ , while results with  $P \leq 0.10$  were considered as potentially statistically significant. These results were not considered statistically significant but could indicate that an effect may be present when for example larger test groups are used. The assumption for normality was almost always met when analyzing the distribution for each treatment combination

group of the dependent variables. Moreover, the homogeneity of variance in the Two-way ANOVA was always met. Welch results were preferred in the one way-ANOVA when homogeneity of variance was not met. In the linear regression the standard residuals were always between -3 and 3. Cook's distance was always lower than 1. Finally, in all the statistical tests outliers when founded were not discarded due to the limited size of the populations.

The function of the different statistical tests is repeated in paragraph 3 for a better understanding of the results.

### 3. Results

The responses of the traits were evaluated by means of the one-way ANOVA to check whether they were different between species under the combined effect of  $Ca$  and  $P_s$ . All traits showed statistically significant differences between the three species as the results of the ANOVA test shows (table 4).

**Table 4. Difference in responses of the different traits under the combined treatment of  $Ca$  and  $P_s$  between the three species (*H.lanatus*, *P.miliaceum* and *S.dulcamara*).**

Traits	B <sub>t</sub>	B <sub>a</sub> :B <sub>b</sub>	LMR	SLA	LAR	N <sub>leaf</sub>	P <sub>leaf</sub>
Species	***	***	***	*	***	*	***

ns = not significant + =  $P \leq 0,10$  \* =  $P \leq 0,05$  \*\* =  $P \leq 0,01$  \*\*\* =  $P \leq 0,001$

More specifically, differences in responses between the three species combinations were investigated by means of the post hoc Tukey's HSD test (table 5).

**Table 5. Difference in responses of the different traits under the combined treatment of  $Ca$  and  $P_s$  between the three species combinations (*H.lanatus-S.dulcamara*, *P.miliaceum - H.lanatus*, *S.dulcamara - P.miliaceum*).**

Traits	Species		
B <sub>t</sub>	<i>H.lanatus-S.dulcamara</i>	<i>P.miliaceum-H.lanatus</i>	<i>S.dulcamara-P.miliaceum</i>
	***	***	n.s
B <sub>a</sub> :B <sub>b</sub>	<i>H.lanatus-S.dulcamara</i>	<i>P.miliaceum-H.lanatus</i>	<i>S.dulcamara-P.miliaceum</i>
	***	***	***
LMR	<i>H.lanatus-S.dulcamara</i>	<i>P.miliaceum-H.lanatus</i>	<i>S.dulcamara-P.miliaceum</i>
	**	***	***
SLA	<i>H.lanatus-S.dulcamara</i>	<i>P.miliaceum-H.lanatus</i>	<i>S.dulcamara-P.miliaceum</i>
	**	*	n.s
LAR	<i>H.lanatus-S.dulcamara</i>	<i>P.miliaceum-H.lanatus</i>	<i>S.dulcamara-P.miliaceum</i>
	n.s	***	***
N <sub>leaf</sub>	<i>H.lanatus-S.dulcamara</i>	<i>P.miliaceum-H.lanatus</i>	<i>S.dulcamara-P.miliaceum</i>
	*	n.s	n.s
P <sub>leaf</sub>	<i>H.lanatus-S.dulcamara</i>	<i>P.miliaceum-H.lanatus</i>	<i>S.dulcamara-P.miliaceum</i>
	n.s	***	***

ns = not significant + =  $P \leq 0,10$  \* =  $P \leq 0,05$  \*\* =  $P \leq 0,01$  \*\*\* =  $P \leq 0,001$

Because statistically significant differences in all traits responses were found between the three species and often also between pairs of species, species were analyzed independently. The results are displayed using boxplots that show the changing trends along all the six treatment combinations for each trait investigated. Each boxplot is associated with two tables presenting the results of the other two one-way ANOVAs. The tables show the significance level of the responses under stepwise  $Ca$  increases (150-450 ppm, 450-800 ppm) at the two  $P_s$  (low, high) and under changing  $P_s$  (high-low) at the three  $Ca$  (150, 450, 800 ppm). Significance level of the responses to rising  $Ca$  and low  $P_s$  and their interaction, calculated by means of the two-way ANOVA are also presented. In section 3.4, the relationships of N<sub>leaf</sub> with  $V_{cmax}$  and of P<sub>leaf</sub> with  $J_{max}$  of *H.lanatus* and *S.dulcamara* were calculated using an ecological linear regression all along the six treatment

combinations. Moreover, the combined effect of  $Ca$  and  $P_s$  on  $V_{cmax}$  and  $J_{max}$  was compared to the combined effect on  $N_{leaf}$  and  $P_{leaf}$ .

In general, the different traits responded more to rising  $Ca$  in the transition from low to ambient level than from ambient to high level, as the results of the post hoc Tukey's HSD test show (table 6).

**Table 6. Effects of the step-wise  $Ca$  increases from low to ambient  $Ca$  (150-450 ppm) and from ambient to high  $Ca$  (450-800 ppm) on the different traits in the three species (*H.lanatus*, *S.dulcamara* and *P.miliaceum*).**

Traits	<i>H.lanatus</i>		<i>S.dulcamara</i>		<i>P.miliaceum</i>	
	<i>Ca</i>		<i>Ca</i>		<i>Ca</i>	
	150-450	450-800	150-450	450-800	150-450	450-800
$B_t$	***	n.s	n.s	*	***	**
$B_a:B_b$	***	n.s	***	**	***	***
LMR	***	n.s	***	n.s	n.s	*
SLA	n.s	*	***	n.s	n.s	n.s
LAR	**	**	n.s	n.s	n.s	+
$N_{leaf}$	**	n.s	***	n.s	***	n.s
$P_{leaf}$	***	n.s	***	n.s	***	+

ns = not significant + =  $P \leq 0,10$  \* =  $P \leq 0,05$  \*\* =  $P \leq 0,01$  \*\*\* =  $P \leq 0,001$

The results per individual of all traits in the three species together with the results of  $V_{cmax}$  and  $J_{max}$  from Rietveld (2016) in *H.lanatus* and *S.dulcamara*, are listed in appendix B.

### 3.1 Biomass growth and biomass allocation

#### *Total biomass ( $B_t$ )*

$B_t$  responded similarly to the combined effect of  $Ca$  and  $P_s$  between the three species (Figure 1). The independent and interactive effects of the external factors were always statistically significant in the three species ( $P \leq 0,001$ ).  $B_t$  showed a statistical significant increase in the transition from low to ambient  $Ca$  in both  $P_s$  levels in all species (table 7; figure 1). It also statistically significantly increased in the transition from ambient to high  $Ca$  under high  $P_s$  (ibid). By contrast, under low  $P_s$  it responded differently between the three species; it slightly decreased in *H.lanatus*, it showed no response in *S.dulcamara* and statistically significantly increased in *P.miliaceum* (ibid).  $B_t$  was generally higher under high  $P_s$  than under low  $P_s$ , apart from *H.lanatus* and *P.miliaceum* at low  $Ca$  where there was no response to  $P_s$  (table 8; figure 1). The difference in  $B_t$  between  $P_s$  levels slightly increased in the transition from low to high  $Ca$  in both *H.lanatus* and *S.dulcamara* (figure 1). Indeed,  $B_t$  increased more under high  $P_s$  than under low  $P_s$  along the  $Ca$  transition (ibid).

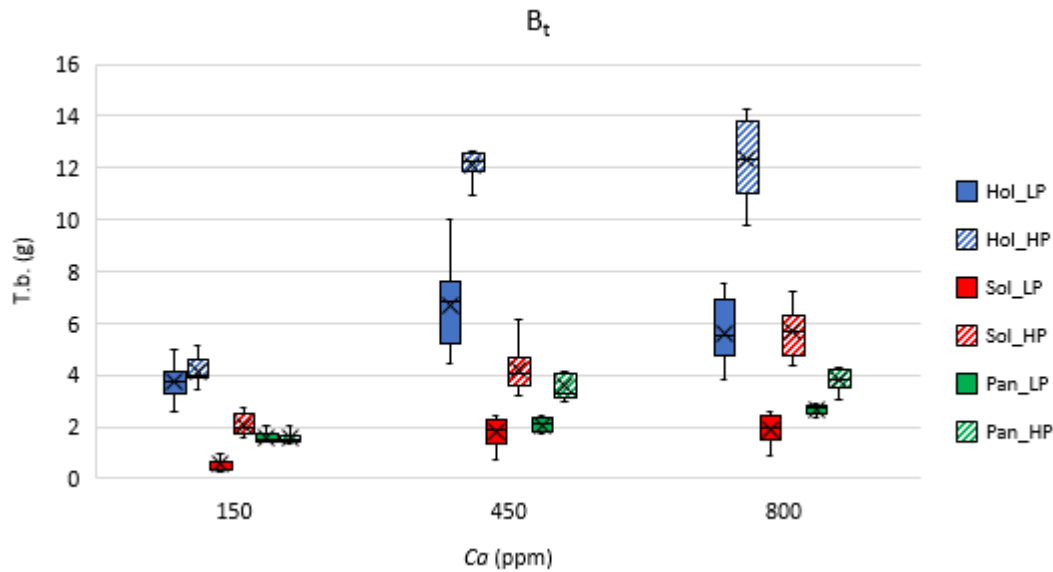


Figure 1. Boxplots representing the combined effect of  $Ca$  (150-450-800 ppm) and  $P_s$  (LP-HP) on  $B_t$  in *H.lanatus* (Hol), *S.dulcamara* (Sol) and *P.miliaceum* (Pan).

Table 7. Effects of the step-wise  $Ca$  increases from low to ambient  $Ca$  (150-450 ppm) and from ambient to high  $Ca$  (450-800 ppm) on  $B_t$  under the two  $P_s$  (low, high) in the three species (*H.lanatus*, *S.dulcamara* and *P.miliaceum*).

Species	$Ca$	$P_s$	
		low	high
<i>H. Lanatus</i>	150-450	***	***
	450-800	+	*
<i>S. Dulcamara</i>	150-450	***	***
	450-800	n.s	**
<i>P. Miliaceum</i>	150-450	**	***
	450-800	***	*

ns = not significant + =  $P \leq 0,10$  \* =  $P \leq 0,05$  \*\* =  $P \leq 0,01$  \*\*\* =  $P \leq 0,001$

Table 8. Effects of  $P_s$  (H-L) on  $B_t$  at the three levels of  $Ca$  (150, 450, 800 ppm) in the three species (*H.lanatus*, *S.dulcamara* and *P.miliaceum*).

Species	$P_s$	$Ca$		
		150	450	800
<i>H. Lanatus</i>	H-L	n.s	***	***
<i>S. Dulcamara</i>	H-L	***	***	***
<i>P. Miliaceum</i>	H-L	n.s	***	***

ns = not significant + =  $P \leq 0,10$  \* =  $P \leq 0,05$  \*\* =  $P \leq 0,01$  \*\*\* =  $P \leq 0,001$

#### Above-below ground biomass ratio ( $B_a:B_b$ )

$B_a:B_b$  responded differently to the combined effect of  $Ca$  and  $P_s$  between the three species (figure 2). The independent effects of these external factors were statistically significant in all species ( $P \leq 0,01$ ), while the interactive effect was statistically significant only in *H.lanatus* and *P.miliaceum* ( $P \leq 0,05$ ). In *H.lanatus*,  $B_a:B_b$  showed a statistically significant decrease in the transition from low to ambient  $Ca$  under both  $P_s$  levels, while showing no response in the transition from ambient to high  $Ca$  (table 9; figure 2).  $B_a:B_b$  was also statistically significantly greater under high  $P_s$  than under low

$P_s$  at low  $Ca$ , while at ambient and high  $Ca$  it did not respond to a change in  $P_s$  (table 10; figure 2). By contrast, in *S.dulcamara* it did not respond to the transition from low to ambient  $Ca$  under both  $P_s$  levels (table 9; figure 2). Differently, it slightly and statistically significantly increased in the transition from ambient to high  $Ca$  under high  $P_s$ , while it showed no response under low  $P_s$  (ibid). It also showed a statistically significant increase under high  $P_s$  compared to low  $P_s$  at ambient and high  $Ca$  but it did not show any response to low  $P_s$  at low  $Ca$  (table 10; figure 2). In *P.miliaceum*, it statistically significantly increased in the transition from low to ambient  $Ca$  under high  $P_s$  before to stop responding, while under low  $P_s$  it showed a statistically significant increase in the transition from low to ambient  $Ca$  before to statistically significantly decrease in the transition from ambient to high  $Ca$  (table 9; figure 2). Finally, it statistically significantly increased under low  $P_s$  compared with high  $P_s$  at low and ambient  $Ca$  while it showed no responses at high  $Ca$  (table 10; figure 2).

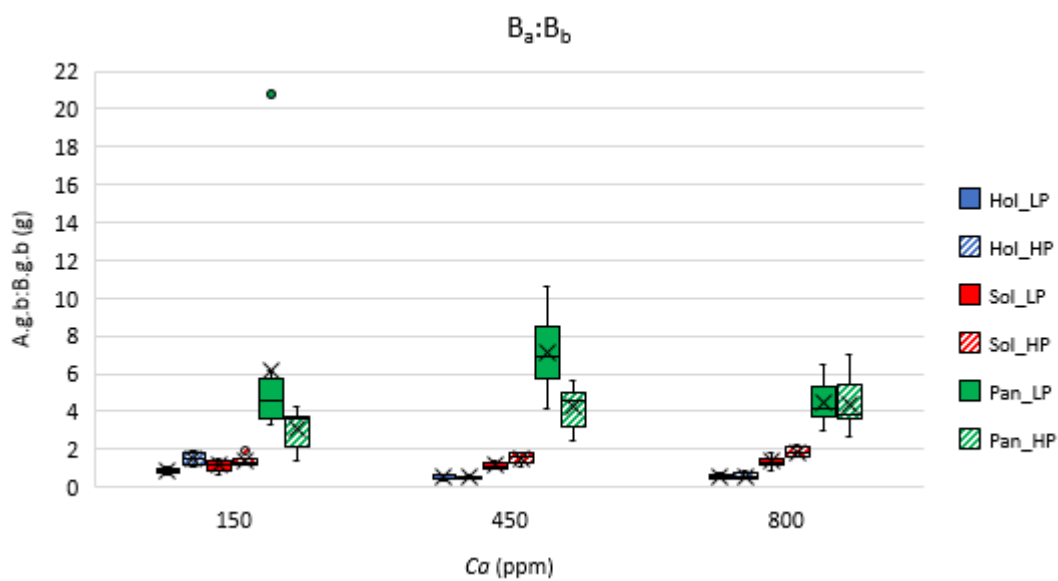


Figure 2. Boxplots representing the combined effect of  $Ca$  (150-450-800 ppm) and  $P_s$  (LP-HP) on  $B_a:B_b$  in *H.lanatus* (Hol), *S.dulcamara* (Sol) and *P.miliaceum* (Pan).

Table 9- Effects of the step-wise  $Ca$  increases from low to ambient  $Ca$  (150-450 ppm) and from ambient to high  $Ca$  (450-800 ppm) on  $B_a:B_b$  under the two  $P_s$  (low, high) in the three species (*H.lanatus*, *S.dulcamara* and *P.miliaceum*).

Species	Ca	$P_s$	
		low	high
<i>H. lanatus</i>	150-450	**	*
	450-800	n.s	n.s
<i>S. dulcamara</i>	150-450	n.s	n.s
	450-800	n.s	+
<i>P. miliaceum</i>	150-450	**	*
	450-800	**	n.s

ns = not significant + =  $P \leq 0,10$  \* =  $P \leq 0,05$  \*\* =  $P \leq 0,01$  \*\*\* =  $P \leq 0,001$

Table 10. Effects of P<sub>s</sub> (H-L) on B<sub>a</sub>:B<sub>b</sub> at the three levels of Ca (150, 450, 800 ppm) in the three species (*H.lanatus*, *S.dulcamara* and *P.miliaceum*).

Species	P <sub>s</sub>	Ca		
		150	450	800
<i>H. lanatus</i>	H-L	***	n.s	n.s
<i>S. dulcamara</i>	H-L	n.s	**	**
<i>P. miliaceum</i>	H-L	**	**	n.s

ns = not significant + =  $P \leq 0,10$  \* =  $P \leq 0,05$  \*\* =  $P \leq 0,01$  \*\*\* =  $P \leq 0,001$

### Leaf mass ratio (LMR)

LMR also responded differently between the three species to the combined effect of Ca and P<sub>s</sub> (figure 3). The independent effect of Ca was statistically significant in all species ( $P \leq 0,001$ ) while the independent effect of P<sub>s</sub> and its interactive effect with Ca were statistically significant only in *H.lanatus* and *P.miliaceum* ( $P \leq 0,01$ ). In *H.lanatus*, LMR showed a statistically significant decrease in the transition from low to ambient Ca under both low and high P<sub>s</sub> (table 11; figure 3). By contrast, in *S.dulcamara*, it statistically significantly increased in the same Ca transition under high P<sub>s</sub> (ibid). In *P.miliaceum*, it once again statistically significantly increased in the same Ca transition under high P<sub>s</sub> (ibid). LMR also significantly changed between P<sub>s</sub> levels at low Ca in *H.lanatus* and at ambient and high Ca in *P.miliaceum*, with values higher under high P<sub>s</sub> than under low P<sub>s</sub> (table 12; figure 3). LMR showed no other statistically significant responses to the combination of Ca and P<sub>s</sub> in the three species (table 11; table 12).

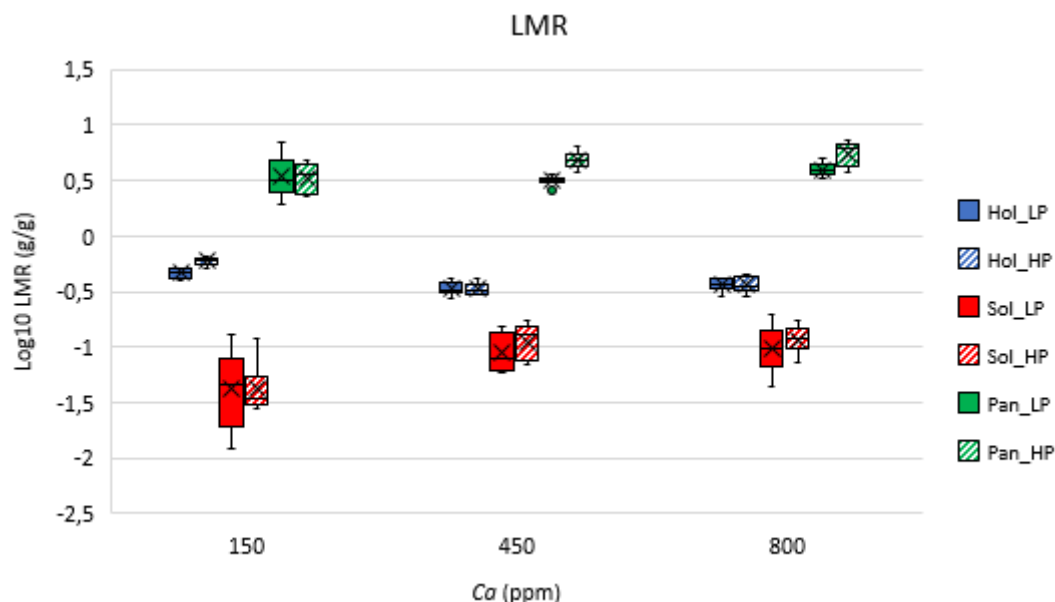


Figure 3. Boxplots representing the combined effect of Ca (150-450-800 ppm) and P<sub>s</sub> (LP-HP) on LMR in *H.lanatus* (Hol), *S.dulcamara* (Sol) and *P.miliaceum* (Pan). Values of LMR were log<sub>10</sub> transformed to better visualize the results of the three species in the same graph.

**Table 11. Effects of the step-wise *Ca* increases from low to ambient *Ca* (150-450 ppm) and from ambient to high *Ca* (450-800 ppm) on LMR under the two *P<sub>s</sub>* (low, high) in the three species (*H.lanatus*, *S.dulcamara* and *P.miliaceum*).**

Species	<i>Ca</i>	<i>P<sub>s</sub></i>	
		low	high
<i>H. lanatus</i>	150-450	***	**
	450-800	n.s	+
<i>S. dulcamara</i>	150-450	n.s	**
	450-800	n.s	n.s
<i>P. miliaceum</i>	150-450	n.s	**
	450-800	n.s	+

*ns* = not significant + =  $P \leq 0,10$  \* =  $P \leq 0,05$  \*\* =  $P \leq 0,01$  \*\*\* =  $P \leq 0,001$

**Table 12. Effects of *P<sub>s</sub>* (H-L) on LMR at the three levels of *Ca* (150, 450, 800 ppm) in the three species (*H.lanatus*, *S.dulcamara* and *P.miliaceum*).**

Species	<i>P<sub>s</sub></i>	<i>Ca</i>		
		150	450	800
<i>H. lanatus</i>	H-L	***	n.s	n.s
<i>S. dulcamara</i>	H-L	n.s	n.s	n.s
<i>P. miliaceum</i>	H-L	n.s	***	**

*ns* = not significant + =  $P \leq 0,10$  \* =  $P \leq 0,05$  \*\* =  $P \leq 0,01$  \*\*\* =  $P \leq 0,001$

### 3.2 *A<sub>leaf</sub>* in relation to biomass

#### *Specific leaf area (SLA)*

SLA responded differently to the combined effect of *Ca* and *P<sub>s</sub>* between the three species (figure 4). The independent effect of *Ca* was statistically significant only in *H.lanatus* and *S.dulcamara* ( $P \leq 0,05$ ) and the independent effect of *P<sub>s</sub>* was statistically significant only in *P.miliaceum* ( $P \leq 0,05$ ). Differently, the interactive effect of the external factors was never statistically significant in all species ( $P \geq 0,1$ ). In *H.lanatus*, SLA increased in the transition from ambient to high *Ca* under low *P<sub>s</sub>*, although with a weak significance level, (table 13; figure 4). In *S.dulcamara*, it showed a statistically significant decrease in the transition from low to ambient *Ca* under both *P<sub>s</sub>* levels (ibid). In *P.miliaceum*, it statistically significantly increased under low *P<sub>s</sub>* compared with high *P<sub>s</sub>*, at high *Ca* (table 14; figure 4). SLA showed no other statistically significant responses to the combinations of *Ca* and *P<sub>s</sub>* in the three species (table 13; table 14).



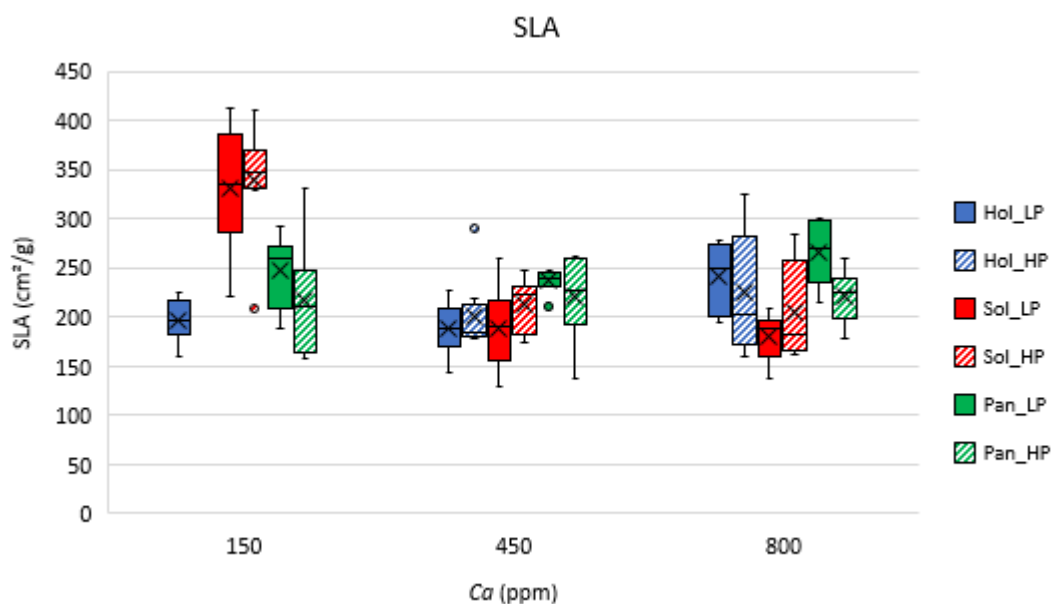


Figure 4. Boxplots representing the combined effect of Ca (150-450-800 ppm) and P<sub>s</sub> (LP-HP) on SLA in *H.lanatus* (Hol), *S.dulcamara* (Sol) and *P.miliaceum* (Pan).

Table 13. Effects of the step-wise Ca increases from low to ambient Ca (150-450 ppm) and from ambient to high Ca (450-800 ppm) on SLA under the two P<sub>s</sub> (low, high) in the three species (*H.lanatus*, *S.dulcamara* and *P.miliaceum*).

Species	Ca	P <sub>s</sub>	
		low	high
<i>H. lanatus</i>	150-450	n.s	-
	450-800	+	n.s
<i>S. dulcamara</i>	150-450	***	***
	450-800	n.s	n.s
<i>P. miliaceum</i>	150-450	n.s	n.s
	450-800	n.s	n.s

ns = not significant + =  $P \leq 0,10$  \* =  $P \leq 0,05$  \*\* =  $P \leq 0,01$  \*\*\* =  $P \leq 0,001$

Table 14. Effects of P<sub>s</sub> (H-L) on SLA at the three levels of Ca (150, 450, 800 ppm) in the three species (*H.lanatus*, *S.dulcamara* and *P.miliaceum*).

Species	P <sub>s</sub>	Ca		
		150	450	800
<i>H. lanatus</i>	H-L	-	n.s	n.s
<i>S. dulcamara</i>	H-L	n.s	n.s	n.s
<i>P. miliaceum</i>	H-L	n.s	n.s	*

ns = not significant + =  $P \leq 0,10$  \* =  $P \leq 0,05$  \*\* =  $P \leq 0,01$  \*\*\* =  $P \leq 0,001$

### Leaf area ratio (LAR)

LAR also responded differently to the combined effect of *Ca* and  $P_s$  between the three species (figure 5). The independent effect of *Ca* was statistically significant only in *H.lanatus* and *P.miliaceum* ( $P \leq 0,05$ ). The independent effect of  $P_s$  and its interactive effect with *Ca* was statistically significant only in *P.miliaceum* ( $P \leq 0,05$ ). In *H.lanatus* LAR showed a statistically significant decrease in the transition from low to ambient *Ca* under low  $P_s$  while, it statistically significantly increased in the same transition under high  $P_s$  in *S.dulcamara* (table 15; figure 5). It also showed a statistically significant increase in the transition from ambient to high *Ca* in *H.lanatus*, (ibid) and statistically significantly changed between  $P_s$  levels at both ambient and high *Ca* in *P.miliaceum*, with values higher under high  $P_s$  than under low  $P_s$  (table 16; figure 5). LAR showed no other statistically significant responses to the combination of *Ca* and  $P_s$  in the three species (table 15; table 16).

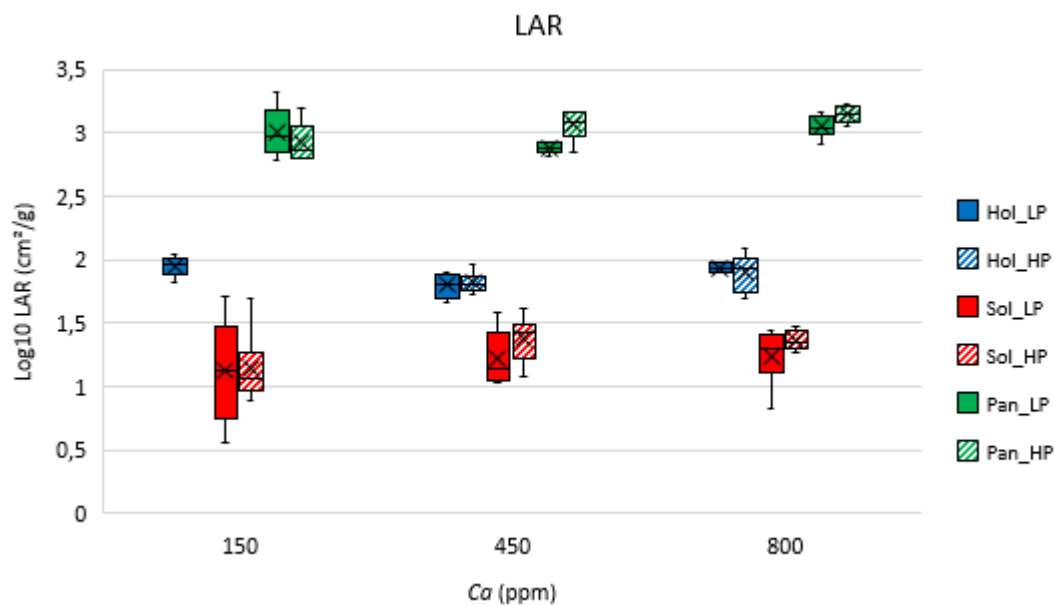


Figure 5. Boxplots representing the combined effect of *Ca* (150-450-800 ppm) and  $P_s$  (LP-HP) on LAR in *H.lanatus* (Hol), *S.dulcamara* (Sol) and *P.miliaceum* (Pan). Values of LAR were log10 transformed to better visualize the results of the three species in the same graph.

Table 15. Effects of the step-wise *Ca* increases from low to ambient *Ca* (150-450 ppm) and from ambient to high *Ca* (450-800 ppm) on LAR under the two  $P_s$  (low, high) in the three species (*H.lanatus*, *S.dulcamara* and *P.miliaceum*).

Species	<i>Ca</i>	$P_s$	
		low	high
<i>H. lanatus</i>	150-450	**	-
	450-800	*	n.s
<i>S. dulcamara</i>	150-450	n.s	**
	450-800	n.s	n.s
<i>P. miliaceum</i>	150-450	n.s	n.s
	450-800	n.s	n.s

ns = not significant + =  $P \leq 0,10$  \* =  $P \leq 0,05$  \*\* =  $P \leq 0,01$  \*\*\* =  $P \leq 0,001$

Table 16. Effects of P<sub>s</sub> (H-L) on LAR at the three levels of Ca (150, 450, 800 ppm) in the three species (*H.lanatus*, *S.dulcamara* and *P.miliaceum*).

Species	P <sub>s</sub>	Ca		
		150	450	800
<i>H. lanatus</i>	H-L	-	n.s	n.s
<i>S. dulcamara</i>	H-L	n.s	n.s	n.s
<i>P. miliaceum</i>	H-L	n.s	*	*

ns = not significant + =  $P \leq 0,10$  \* =  $P \leq 0,05$  \*\* =  $P \leq 0,01$  \*\*\* =  $P \leq 0,001$

### 3.3 Leaf nutrients

#### Concentration of nitrogen in the leaf ( $N_{leaf}$ )

$N_{leaf}$  responded similarly to the combined effect of Ca and P<sub>s</sub> between the three species (figure 6). The independent effects of Ca and P<sub>s</sub> were statistically significant in all three species ( $P \leq 0,05$ ) while their interactive effect was statistically significant only in *P.miliaceum* ( $P \leq 0,001$ ). Under high P<sub>s</sub>  $N_{leaf}$  showed a statistically significant decrease in the transition from low to ambient Ca in all species (table 17; figure 6). By contrast, under low P<sub>s</sub>, it decreased in the same Ca transition only in *H.lanatus* and *S.dulcamara*, although showing statistical significance only in *S.dulcamara*, while in *P.miliaceum* it showed no response (ibid).  $N_{leaf}$  showed no statistically significant responses in the transition from ambient to high Ca (ibid). The responses of  $N_{leaf}$  under changing P<sub>s</sub> were statistically significant in all species at all Ca levels, apart from *S.dulcamara* at low Ca (table 18; figure 6).  $N_{leaf}$  was generally higher under low P<sub>s</sub> than under high P<sub>s</sub>, apart from *P.miliaceum* at low Ca where it experienced the opposite trend (ibid).

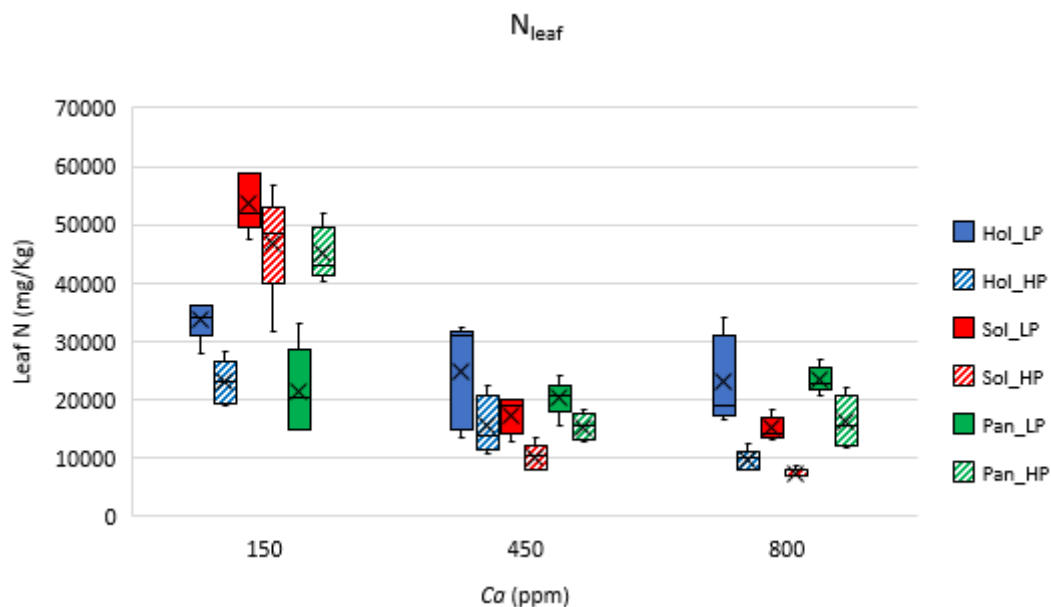


Figure 6. Boxplots representing the combined effect of Ca (150-450-800 ppm) and P<sub>s</sub> (LP-HP) on  $N_{leaf}$  | *H.lanatus* (Hol), *S.dulcamara* (Sol) and *P.miliaceum* (Pan).

**Table 17. Effects of the step-wise *Ca* increases from low to ambient *Ca* (150-450 ppm) and from ambient to high *Ca* (450-800 ppm) on  $N_{leaf}$  under the two  $P_s$  (low, high) in the three species (*H.lanatus*, *S.dulcamara* and *P.miliaceum*).**

Species	<i>Ca</i>	$P_s$	
		low	high
<i>H. lanatus</i>	150-450	n.s	***
	450-800	n.s	n.s
<i>S. dulcamara</i>	150-450	***	***
	450-800	n.s	n.s
<i>P. miliaceum</i>	150-450	n.s	***
	450-800	n.s	n.s

*ns* = not significant + =  $P \leq 0,10$  \* =  $P \leq 0,05$  \*\* =  $P \leq 0,01$  \*\*\* =  $P \leq 0,001$

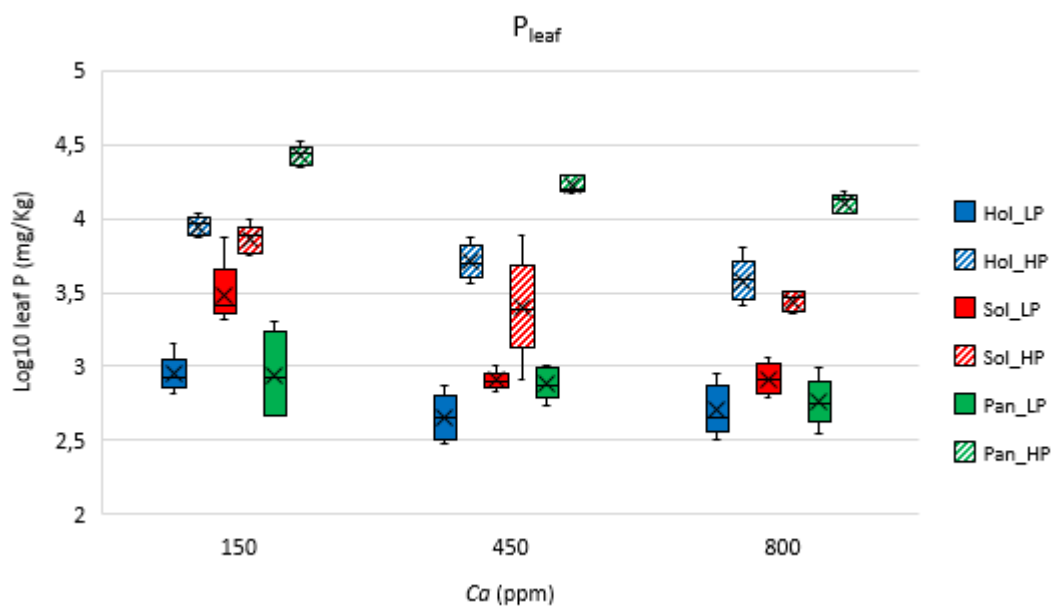
**Table 18. Effects of  $P_s$  (H-L) on  $N_{leaf}$  at the three levels of *Ca* (150, 450, 800 ppm) in the three species (*H.lanatus*, *S.dulcamara* and *P.miliaceum*).**

Species	$P_s$	<i>Ca</i>		
		150	450	800
<i>H. lanatus</i>	H-L	***	+	**
<i>S. dulcamara</i>	H-L	n.s	**	***
<i>P. miliaceum</i>	H-L	***	*	**

*ns* = not significant + =  $P \leq 0,10$  \* =  $P \leq 0,05$  \*\* =  $P \leq 0,01$  \*\*\* =  $P \leq 0,001$

#### *Concentration of phosphorus in the leaf ( $P_{leaf}$ )*

$P_{leaf}$  responded similarly to the combined effect of *Ca* and  $P_s$  between the three species (figure 7). The independent effects of both *Ca* and  $P_s$  were statistically significant in all species ( $P \leq 0,001$ ), while their interactive effects were statistically significant only in *H.lanatus* and *P.miliaceum* ( $P \leq 0,001$ ).  $P_{leaf}$  showed a statistically significant decrease in the transition from low to ambient *Ca*, similarly to  $N_{leaf}$ , under both  $P_s$  levels (table 19; figure 7). The only exception was in *P.miliaceum*, where under low  $P_s$  no responses could be detected (ibid). By contrast, there was no statistically significant change in  $P_{leaf}$  in the transition from ambient to high *Ca* (ibid). The  $P_{leaf}$  responses under changing  $P_s$  were always statistically significant in the three species, with values higher under high  $P_s$  than under low  $P_s$  (table 20; figure 7).



**Figure 7. Boxplots representing the combined effect of  $Ca$  (150-450-800 ppm) and  $P_s$  (LP-HP) on  $P_{leaf}$  in *H.lanatus* (Hol), *S.dulcamara* (Sol) and *P.miliaceum* (Pan). Values of LMR were log10 transformed to better visualize the results of the three species in the same graph.**

**Table 19. Effects of the step-wise  $Ca$  increases from low to ambient  $Ca$  (150-450 ppm) and from ambient to high  $Ca$  (450-800 ppm) on  $P_{leaf}$  under the two  $P_s$  (low, high) in the three species (*H.lanatus*, *S.dulcamara* and *P.miliaceum*).**

Species	$Ca$	$P_s$	
		low	High
<i>H. lanatus</i>	150-450	*	***
	450-800	n.s	n.s
<i>S. dulcamara</i>	150-450	*	**
	450-800	n.s	n.s
<i>P. miliaceum</i>	150-450	n.s	***
	450-800	n.s	n.s

ns = not significant + =  $P \leq 0,10$  \* =  $P \leq 0,05$  \*\* =  $P \leq 0,01$  \*\*\* =  $P \leq 0,001$

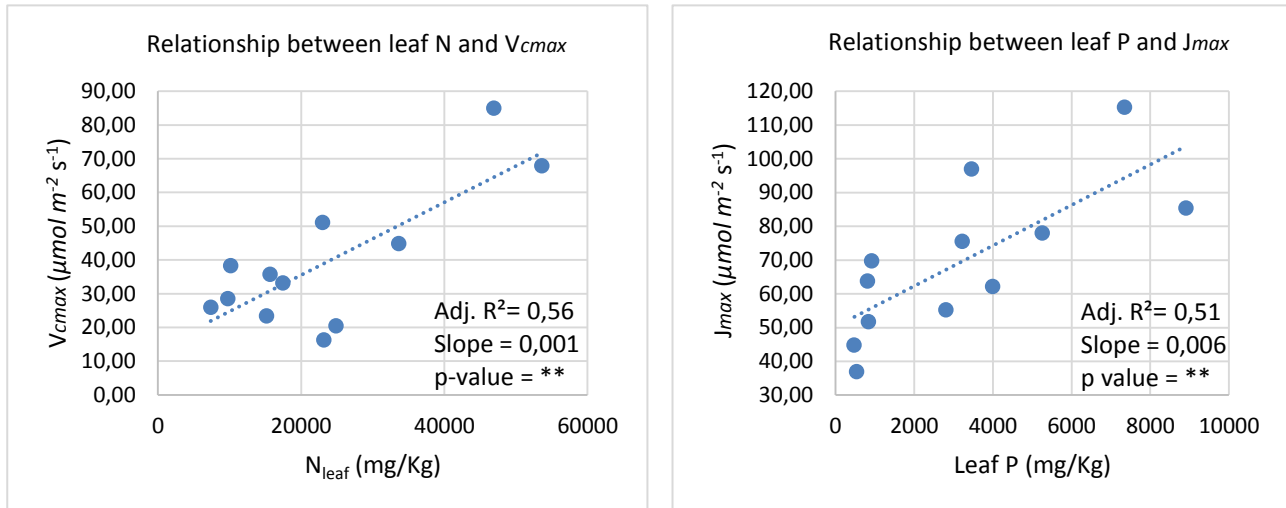
**Table 20. Effects of  $P_s$  (H-L) on  $P_{leaf}$  at the three levels of  $Ca$  (150, 450, 800 ppm) in the three species (*H.lanatus*, *S.dulcamara* and *P.miliaceum*).**

Species	$P_s$	$Ca$		
		150	450	800
<i>H. lanatus</i>	H-L	***	**	***
<i>S. dulcamara</i>	H-L	**	*	***
<i>P. miliaceum</i>	H-L	***	***	***

ns = not significant + =  $P \leq 0,10$  \* =  $P \leq 0,05$  \*\* =  $P \leq 0,01$  \*\*\* =  $P \leq 0,001$

### 3.4 Leaf nutrients and their relationships with photosynthetic traits

$V_{cmax}$  and  $J_{max}$  were positively and statistically significantly related with  $N_{leaf}$  and  $P_{leaf}$ , respectively, since the p-value of their linear regressions was statistically significant and the data well fitted the regression line (figure 8). However, a change in  $P_{leaf}$  resulted in a larger change in  $J_{max}$  (slope = 0,006) than a change in  $N_{leaf}$  in  $V_{cmax}$  (slope = 0,001).



**Figure 8.** Linear regression lines between  $N_{leaf}$  and  $V_{cmax}$  (left graph) and  $P_{leaf}$  and  $J_{max}$  (right graph) using averages of populations for each treatment combination of *H.lanatus* and *S.dulcamara*. Graphs display relative adjusted  $R^2$ , slope and p-value with \*\* =  $P \leq 0,01$ .

$P_{leaf}$  and  $J_{max}$  responded similarly between each other under the combined effect of  $C_a$  and  $P_s$  while  $N_{leaf}$  and  $V_{cmax}$  responded differently between each other (figure 9). The independent effects of  $C_a$  and  $P_s$  were statistically significant in all traits ( $N_{leaf}$ ,  $P_{leaf}$ ,  $V_{cmax}$  and  $J_{max}$ ) ( $P \leq 0,05$ ) while their interactive effect was statistically significant only in  $P_{leaf}$  ( $P \leq 0,01$ ). Both  $N_{leaf}$  and  $V_{cmax}$  showed a statistically significant decrease in the transition from low to ambient  $C_a$ , while there was no statistically significant response in the transition from ambient to high  $C_a$  (table 21; figure 9). Similarly, both  $P_{leaf}$  and  $J_{max}$  decreased in the transition from low to ambient  $C_a$  and did not responded in the transition from ambient to high  $C_a$ , apart from  $J_{max}$  under high  $P_s$ , which statistically significantly further decreased in the latter  $C_a$  transition (ibid). Both  $P_{leaf}$  and  $J_{max}$  show a statistically significant further decrease under low  $P_s$  compared with high  $P_s$  (table 22; figure 9). Differently,  $N_{leaf}$  and  $V_{cmax}$  responded oppositely between each other, with  $N_{leaf}$  increasing and  $V_{cmax}$  decreasing under low  $P_s$  at ambient and high  $C_a$  (ibid). However, both showed no response under low  $P_s$  at low  $C_a$  (ibid). Finally, the effect of low  $P_s$  on  $P_{leaf}$  was reduced in the transition from low to ambient  $C_a$  while was increased on  $J_{max}$  in the same  $C_a$  transition (figure 9).

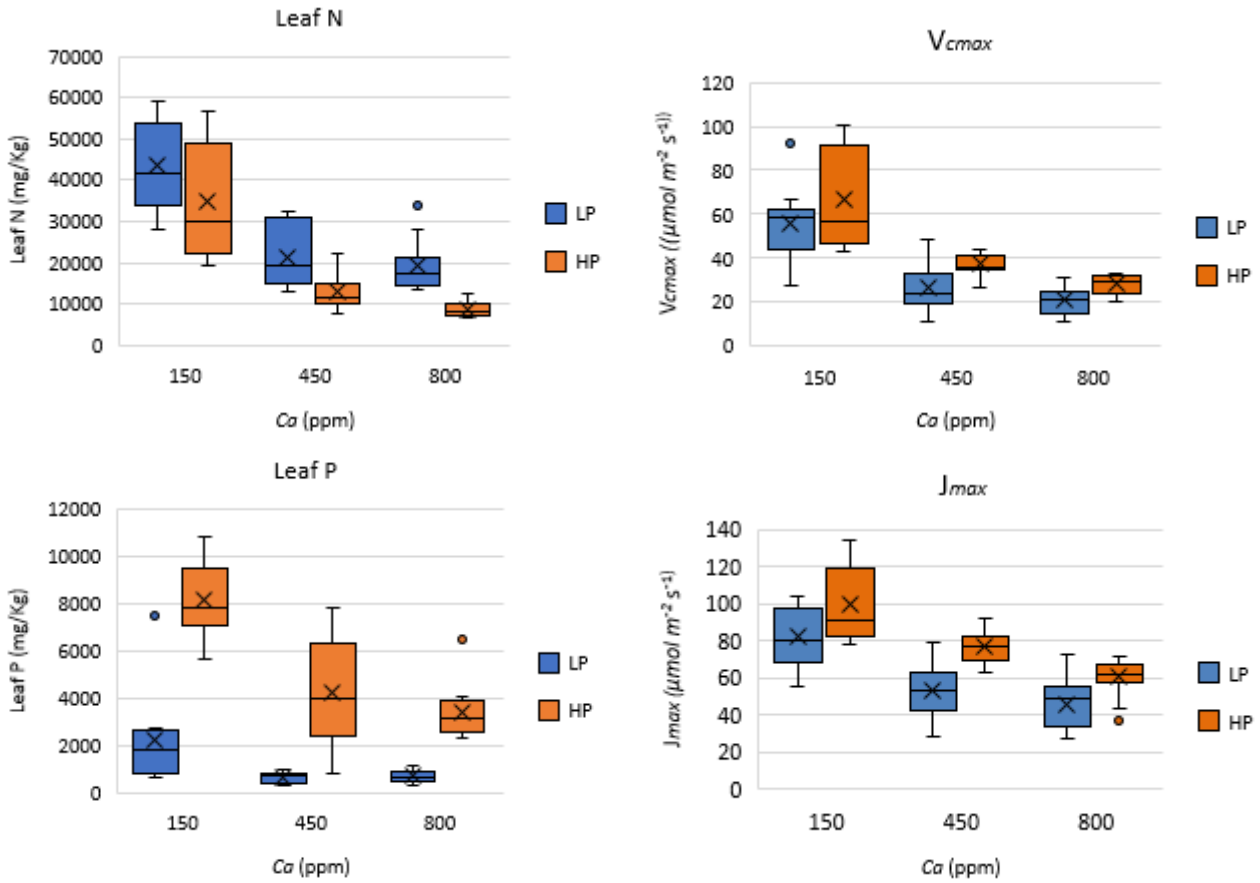


Figure 9. Boxplots representing the combined effect of  $Ca$  (150-450-800 ppm) and  $P_s$  (LP-HP) on  $N_{leaf}$  (top left graph),  $V_{cmax}$  (top right graph),  $P_{leaf}$  (bottom left graph) and  $J_{max}$  (bottom right graph) using averages of populations for each treatment combination of *H.lanatus* and *S.dulcamara*.

Table 21. Effects of the step-wise  $Ca$  increases from low to ambient  $Ca$  (150-450 ppm) and from ambient to high  $Ca$  (450-800 ppm) on  $N_{leaf}$ ,  $P_{leaf}$ ,  $V_{cmax}$  and  $J_{max}$  under the two  $P_s$  (low, high) for grouped *H.lanatus* and *S.dulcamara* data.

Traits	$Ca$	$P_s$	
		low	high
$N_{leaf}$	150-450	***	***
	450-800	n.s	n.s
$V_{cmax}$	150-450	***	***
	450-800	n.s	n.s
$J_{max}$	150-450	***	***
	450-800	n.s	**
$P_{leaf}$	150-450	*	***
	450-800	n.s	n.s

ns = not significant + =  $P \leq 0,10$  \* =  $P \leq 0,05$  \*\* =  $P \leq 0,01$  \*\*\* =  $P \leq 0,001$

**Table 22. Effects of  $P_s$  (H-L) on  $N_{leaf}$ ,  $P_{leaf}$ ,  $V_{cmax}$  and  $J_{max}$  at the three levels of  $Ca$  (150, 450, 800 ppm) for grouped *H.lanatus* and *S.dulcamara* data.**

Traits	$P_s$	$Ca$		
		150	450	800
$N_{leaf}$	H-L	n.s	***	***
$V_{cmax}$	H-L	n.s	**	**
$J_{max}$	H-L	*	***	**
$P_{leaf}$	H-L	***	***	***

*ns* = not significant + =  $P \leq 0,10$  \* =  $P \leq 0,05$  \*\* =  $P \leq 0,01$  \*\*\* =  $P \leq 0,001$

## 4. Discussion

### 4.1 Response of biomass growth

The first and key aim of the present study was to investigate how NPP and therefore  $B_t$  responded to the combined effect of rising  $Ca$  and low  $P_s$ . Low  $P_s$  was expected to reduce the increasing  $B_t$  to rising  $Ca$  (Conroy et al.,1988; Curtis & Wang,1998; Goudriaan & De Ruiter,1983; Moorby & Besford,1983; Zangerl & Bazzaz,1984). This hypothesis was confirmed, since the same general trend was observed in all species. Moreover, there was a stronger effect of rising  $Ca$  on  $B_t$  in *H.lanatus* in the transition from low to ambient  $Ca$  than from ambient to future  $Ca$  supporting the results from Baker et al. (1990), Dippery et al. (1995) and Ward et al. (1999). This pattern may indicate that photosynthetic plants responses are better adapted to low rather than high  $Ca$  (Körner,2006; Sage & Cowling,1999; Saxe et al.,1998). In addition, my results show an interactive effect of rising  $Ca$  and low  $P_s$  on  $B_t$  and a common response for this effect was found in *H.lanatus* and *S.dulcamara*. Indeed, in these species  $B_t$  increasingly and statistically significantly reduced  $B_t$  with rising  $Ca$ , suggesting that  $P_s$  scarcity can hamper NPP at the end of the century more strongly than at the present. Apparently, no previous studies reported an interactive effect of rising  $Ca$  and low  $P_s$  on NPP (Wieder et al.,2015).

#### *Potential consequences of low $P_s$ in fully coupled climate-carbon cycle models*

Biomass growth responses to rising  $Ca$  and low  $P_s$  shown in the present study are extremely relevant for fully coupled climate-carbon cycle models. Indeed, current C cycle projections are likely to overestimate the ability of land surface to absorb atmospheric  $CO_2$ , since low  $P_s$  is not integrated in such models (Wieder et. al, 2015) although it can limit plant growth and it is particularly low in many natural ecosystems. Moreover, low P rates of mineralization are not fast enough to meet the increased nutrient demand generated from projected NPP increases (Cleveland et al.,2013). My analysis not only confirms that NPP is statistically significantly reduced by low  $P_s$ , as many studies have already reported (Conroy et al.,1988; Curtis & Wang,1998; Goudriaan & De Ruiter,1983; Moorby & Besford,1983; Zangerl & Bazzaz,1984), but it also indicates that this effect can be stronger at rising  $Ca$ , further reducing NPP. Strong limitation on NPP could results in net terrestrial C losses to the atmosphere, especially in the tropics, contrary to the terrestrial C storage projected in many fully coupled climate-carbon cycle models (Wieder et. al, 2015).



## 4.2 Response of biomass allocation

The present study also shows that plants reallocate biomass between aboveground and below ground tissue depending on  $Ca$ - $P_s$  conditions. These reallocation responses may be an ecophysiological-induced mechanism intended to affect plant carbon assimilation (Callaway et al.,1994). Biomass was expected to be allocated more to the roots than to the above ground section with rising  $Ca$  (Ainsworth & Long,2005; Ceulemans & Mousseau,1994; Eamus & Jarvis,1989), once again with a stronger effect in the transition from low to ambient  $Ca$  than from ambient to high  $Ca$  (Baker et al.,1990), and under low  $P_s$  (Burslem & Turner,1996; Ceulemans & Mousseau,1994; Eamus & Jarvis,1989; Knox & Clarke,2005; Santiago et al.,2012), potentially offsetting carbon photosynthetic gains (Callaway et al.,1994). Thus, low  $P_s$  was expected to further lower both  $B_a:B_b$  and LAR, already decreasing with rising  $Ca$ . However, in the present study different effects of rising  $Ca$  and low  $P_s$  were found between the three species. Indeed, rising  $Ca$  either did not show any effect or in few cases statistically significantly increased both  $B_a:B_b$  and LMR. By contrast,  $P_s$  statistically significantly lowered  $B_a:B_b$  only in *S.dulcamara*, independently from  $Ca$ , and statistically significantly lowered LMR only in *H.lanatus* at low  $Ca$  and in *P.miliaceum* at ambient and high  $Ca$ .

The differing responses of the three species and inconsistency between results and expectations support the study of Poorter and Nagel (2000) which found a high variability in biomass allocation based on various environmental growing conditions, species physiological characteristics and ontogeny. Therefore, it is not possible to draw any general conclusions regarding shifts in plant biomass allocation at the future  $Ca$  increase under low  $P_s$ . Nonetheless, the effect of low  $P_s$ , when present, always lowered LMR, suggesting that, independently from  $Ca$ , less biomass is invested in the leaves under shortage of soil P. Finally, the present study found that a statistically significant interactive effect of  $Ca$  and  $P_s$  was present in *H.lanatus* and *P.miliaceum* on both  $B_a:B_b$  and LMR but no common trends of this effect can be highlighted due to high variability in responses between species. This result cannot again be compared with the literature as no information regarding the interactive effect of the external factors on biomass allocation were found.

## 4.3 Response of $A_{leaf}$ in relation to biomass

The third aim of the present study was to investigate if and how  $A_{leaf}$  changed in relation to  $B_t$  and  $B_{leaf}$  due to the combined effect of rising  $Ca$  and low  $P_s$ . Once again, these morphological changes may be a result of ecophysiological mechanisms affecting carbon assimilation (Evans & Poorter,2001; Hättenschwiler et al.,1997). The present study found a very low statistical significance of SLA and LAR responses. Moreover, as seen for biomass allocation, species adopted very different strategies under the combined effect of low  $P_s$  and rising  $Ca$ , something that was expected for LAR, even if only under low  $P_s$  (Feller et al.,2007) but not for SLA. Nevertheless, several studies reported that  $A_{leaf}$  increases relatively less than  $B_{leaf}$  and than  $B_t$  with rising  $Ca$  (Bazzaz,1990; Centritto & Jarvis,1999; Hättenschwiler & Körner,1997; Hättenschwiler et al.,1997; Rogers et al.,1996; Tissue & Lewis,2010), and that it decreases relatively more than  $B_{leaf}$  and than  $B_t$  under low  $P_s$  (Conroy et al.,1988; Radin & Eidenbock,1984; Tissue & Lewis,2010). Therefore, low

$P_s$  was hypothesized to further reduce SLA and LAR already decreasing with rising  $Ca$ , potentially limiting photosynthetic carbon uptake (Evans & Poorter,2001; Hättenschwiler et al.,1997). In support of these hypotheses, the present study found a statistically significant decrease in SLA but only with rising  $Ca$ , and only in *S.dulcamara*, and a decrease in LAR but only under low  $P_s$  at ambient and high  $Ca$ , and only in *P.miliaceum*. Moreover, a statistically significant interactive effect of rising  $Ca$  and low  $P_s$  was found on LAR, but only once again in *P.miliaceum*, while no statistically significant interactive effect was found on SLA. Similarly, Tissue and Lewis (2010) reported an interactive effect of rising  $Ca$  and low  $P_s$  on LAR and as well no interactive effect on SLA. Nevertheless, in contrast with the results of Tissue and Lewis (2010) no stronger effect of low  $P_s$  on LAR with rising  $Ca$  from low to ambient level was found.

SLA and LAR responses show general inconsistency with the hypotheses, low statistical significance and a high variability between species. As such, as seen for biomass allocation, it is not possible to draw general conclusions on the adaptation of SLA and LAR to a future  $Ca$  increase under low  $P_s$ . However, the low statistical significance of changes in SLA and LAR could mean that plants, when well lighted, do not have to invest in  $A_{leaf}$  (De Groot et al.,2001) regardless of  $Ca$  and  $P_s$ .

#### 4.4 Responses of leaf nutrients and their relationships with photosynthetic traits

The final aim of the present study was to investigate how  $N_{leaf}$  and  $P_{leaf}$  responded to the combined effect of rising  $Ca$  and low  $P_s$ , and how their changes were related to changes in  $V_{cmax}$  and  $J_{max}$ , respectively. My results show that  $N_{leaf}$  and  $P_{leaf}$  responses with rising  $Ca$  were similar between each other, contradicting the results of Tissue and Lewis (2010) which found a stronger  $Ca$  effect on  $P_{leaf}$ , while they were opposite under low  $P_s$ . Moreover, leaf nutrients responses under rising  $Ca$  and low  $P_s$  were rather comparable between species. The only anomaly was presented on  $N_{leaf}$  in *P.miliaceum* at low  $Ca$ , where low  $P_s$  showed an opposite effect in comparison to the general trend. However, the present study analyzed only three species, and as such it is difficult to assess whether or not this was indeed an anomaly.

Both  $N_{leaf}$  and  $P_{leaf}$  were expected to have a positive relationship with  $V_{cmax}$  and  $J_{max}$ , respectively (Domingues et al.,2010; Walker et al.,2014), directly regulating photosynthesis through the allocation of N to RuBisCO (Ainsworth & Rogers,2007; Griffin et al.,2000; Lewis et al.,2004) and P to the ETC (Domingues et al.,2010). My results support these hypotheses, since these positive relationships were found.  $V_{cmax}$  and  $J_{max}$  showed a statistically significant decrease with rising  $Ca$ , more steep in the transition from low to ambient  $Ca$  than from ambient to future  $Ca$ , similarly to  $N_{leaf}$  and  $P_{leaf}$ . Comparable results were reported by Tissue and Lewis (2010). The stronger effect of rising  $Ca$  in the low-ambient  $Ca$  transition may suggest that some ecophysiological plants responses could be better adapted to low rather than high  $Ca$  (Körner,2006; Sage & Cowling,1999; Saxe et al.,1998). Moreover,  $J_{max}$  further statistically significantly decreased under low  $P_s$ , similarly to  $P_{leaf}$ . Differently,  $V_{cmax}$  showed a statistically significant decrease under low  $P_s$  even if only at ambient and high  $Ca$ , while  $N_{leaf}$  statistically significantly increased under the same conditions. Low  $P_s$  was expected to weaken the positive relationship between  $N_{leaf}$  and  $V_{cmax}$  since the two traits were expected to respond differently under low  $P_s$  (Tissue & Lewis,2010) and therefore the

hypothesis can be confirmed. My results could also support the conclusions of Griffin et al. (2000) which indicate that  $N_{\text{leaf}}$  can be reallocated to other photosynthetic or no photosynthetic systems more N limited than RuBisCO. This can be also ascertained from the lack of strength of linear regression (Adj.  $R^2 = 0,56$ ) found in the present study. Comparable conclusions can be made on the relationship between  $P_{\text{leaf}}$  and  $J_{\text{max}}$ , since their Adj.  $R^2$  was equal to 0,51. However,  $P_{\text{leaf}}$  seemed to have stronger effect on  $J_{\text{max}}$  (slope = 0,006) than  $N_{\text{leaf}}$  on  $V_{\text{cmax}}$  (slope = 0,001). Finally, there was an interactive effect of rising  $Ca$  and low  $P_s$  on the relationship between  $P_{\text{leaf}}$  and  $J_{\text{max}}$ . Indeed,  $P_{\text{leaf}}$ , in contrast to  $N_{\text{leaf}}$ , experienced a statistically significant response under the interaction of the external factors. The effect of low  $P_s$  on  $P_{\text{leaf}}$  was milder in the transition from low to ambient  $Ca$  and was related to a larger effect on  $J_{\text{max}}$  in the same  $Ca$  transition. Therefore, plants under low  $P_s$  could suffer a milder reduction in  $P_{\text{leaf}}$  at present  $Ca$  compared with glacial  $Ca$ . As the reduced response of  $P_{\text{leaf}}$  related with larger response of  $J_{\text{max}}$ , the effect of low  $P_s$  seems to weaken also the positive relationship between  $P_{\text{leaf}}$  and  $J_{\text{max}}$ , even if interactively with  $Ca$  and with a milder effect than on the relationship between  $N_{\text{leaf}}$  and  $V_{\text{cmax}}$ . Once again, no comparisons with expectations regarding the interactive effect of rising  $Ca$  and low  $P_s$  on the relationships between leaf nutrients and photosynthetic traits can be made, since, to the best of my knowledge, no previous studies investigated on this.

#### 4.5 Limitations and recommendations for future research

##### *Limitations*

This study suffered some limitations that could be improved in future research, beginning with nutrient solution preparation, continuing with samples size and finishing with data grouping.

The nutrient solution had rather extreme ratios of N:P (45N:1P and 1N:1P) (Cleveland & Liptzin, 2007). More specifically, 1N:1P is a very utmost and very rare condition in natural ecosystems (ibid). A solution containing 1N:1P was erroneously prepared in the experiment of Rietveld (2016). Because the present study was intended to be a continuation of Rietveld's work, no changes to the nutrient solutions were made. Despite the very extreme ratios being good benchmarks for visualizing the effect of low  $P_s$  on plants, it is plausible that the results of this study should be to some extent resized if intended to represent plant responses in common natural ecosystems suffering soil P limitation. Moreover, only P was decreased in the solution, although N could also have been simultaneously increased to consider the ongoing modification of the N cycle. Indeed, nitrogen is well known for affecting NPP and associated photosynthetic and physiological plant responses (LeBauer & Treseder, 2008; McGuire et al., 1995) and its biologically available form in the soil have almost doubled since the industrial revolution because of an anthropogenic alteration of the N cycle (Vitousek et al., 1997).

The samples used were very small, reducing the power of the statistical analysis to detect significance. Indeed, sample populations swung from a minimum of five to a maximum of eight individuals per treatment combination. The decision to consider this small sample sizes was unavoidable since the number of individuals of *H. lanatus* and *S. dulcamara* was already set before the current experiment started; Rietveld grew these species populations and had started

measuring photosynthetic responses of above sample sizes. In addition, the decision for the particularly low number of individuals for  $N_{\text{leaf}}$  and  $P_{\text{leaf}}$  (only five) was a result of the limited budget of the present study which could not cover the running cost of more measurements using the Carlo Erba NA1500 elemental analyzer and the S2 Picofox machine. Moreover,  $V_{\text{cmax}}$ ,  $J_{\text{max}}$ , SLA and LAR sample sizes swung between species because some individuals dried out during the photosynthetic measurements and many leaves were wrinkled at the harvest. For the same latter reason no  $A_{\text{leaf}}$  measurements could be taken for *H.lanatus* in one treatment combination (low  $Ca$ -High  $P_s$ ).

Finally, in the linear regression *H.lanatus* and *S.dulcamara* were grouped together in the same data set even if they showed rather different responses to the combination of rising  $Ca$  and low  $P_s$ . This decision was forced by the fact that the precise leaves used to measure photosynthetic traits were not recorded by Rietveld and therefore it was not possible to continue measuring leaf nutrients on the same leaves. If measurements had been done on the same individuals, a linear regression could have been carried out at individual level and it would have been possible to compare six different linear regressions in each species, one per each treatment combination. Thus, it would have been easier to understand the influence of the external factors on the relationships between leaf nutrients and photosynthetic traits.

### *Recommendations*

Because of the aforementioned limitations, future studies could adopt less extreme nutrient ratios in the solution than the ones adopted in the present study, especially for 1N:1P, to better represent plant responses under soil P limitation in natural ecosystems. In addition, to fully understand the variation in NPP and plants responses to future  $Ca$ , P and N could be simultaneously varied in the solution. Further research could also use a larger sample size of individuals to improve the power of the statistical analysis and make sure to have the same sample size per treatment combination between different species and traits. Finally, linear regressions could be carried out at individual level to better understand the combined effect of the external factors on the relationships between leaf nutrients and photosynthetic traits.

The present study also paves the way for new research directions. First, because the ecophysiological mechanisms underlying altered biomass allocation,  $A_{\text{leaf}}$  in relation to biomass and leaf nutrients remain unknown. Second, because my results only suggest that  $N_{\text{leaf}}$  could be reallocated to other photosynthetic or no photosynthetic systems more N limited than RuBisCO under low  $P_s$ , further studies could investigate the biochemical relationship between  $N_{\text{leaf}}$  and N concentrations in RuBisCO to further support my hypothesis.

## 5. Conclusions

Results of the present study indicate that with rising  $Ca$ , regardless of P nutrition, NPP could increase at a lower pace in the transition to future  $Ca$  compared with how it did in the past, as the responses of *H.lanatus* suggest, presumably indicating that photosynthetic responses are better adapted to low rather than high  $Ca$ . Moreover, as a result of the interactive effect of  $Ca$  and  $P_s$ , low  $P_s$  could hamper NPP at the end of the century more strongly than at the present, accordingly to the responses of *H.lanatus* and *S.dulcamara*. Soil P is already scarce in many ecosystems and due to the low rate of P mineralization it will not meet the increased nutrient demand required by increased photosynthetic rates. Therefore, it is likely that fully coupled climate-carbon cycle model projections misrepresent future plant carbon sequestration since they do not integrate low  $P_s$ . At the same time, in order to adapt to rising  $Ca$  and low  $P_s$  conditions plants applied different ecophysiological mechanisms that in turn can affect photosynthetic carbon gains. Increased biomass allocation to the roots, reduced  $A_{leaf}$  in relation to biomass and reduced  $N_{leaf}$  and  $P_{leaf}$  are often indicated in the literature as some of the results of these ecophysiological mechanisms responsible of the down regulation of NPP. My results showed a high variability in biomass allocation, SLA and LAR responses between species, suggesting that species can adopt very different ecophysiological strategies under the independent and interactive effect of low  $P_s$  and rising  $Ca$ , and therefore no general conclusions can be drawn. However, the effect of low  $P_s$ , independently from  $Ca$ , always lowered LMR, suggesting that less biomass is invested in the leaves under shortage of soil P. Moreover, the low statistical significance of SLA and LAR responses suggests that, if sufficiently lighted, plants no longer need to invest in  $A_{leaf}$  regardless of variations in  $Ca$  and in  $P_s$ . Differently, the present study demonstrated that under the combined effect of rising  $Ca$  and low  $P_s$   $N_{leaf}$  and  $P_{leaf}$  had similar responses between species. Leaf nutrients decreased similarly with rising  $Ca$ , more strongly in the transition from low to ambient than from ambient to high  $Ca$ , independently from the nutrient addition regime, while under low  $P_s$   $N_{leaf}$  increased and  $P_{leaf}$  further decreased. The effect of  $Ca$  may again indicate that also some ecophysiological plant responses may be better adapted to low than to high  $Ca$ . Moreover, because of a statistically significant interactive effect of the external factors  $P_{leaf}$  seems to be reduced to a lesser extent by low  $P_s$  at present  $Ca$  compared with glacial  $Ca$ . My results also support the existence of positive relationships of  $N_{leaf}$  and  $P_{leaf}$  with  $V_{cmax}$  and  $J_{max}$ , respectively, suggesting, according to the literature, a plausible regulation of  $N_{leaf}$  on the allocation of N to RuBisCO and of  $P_{leaf}$  on the allocation of P to ETC. However, both the positive relationships lacked of strength. Moreover, low  $P_s$  weakened these positive relationships, with a stronger effect on the relationship between  $N_{leaf}$  and  $V_{cmax}$ . These results may indicate that  $N_{leaf}$  could be reallocated to other photosynthetic or non photosynthetic systems more N limited than RuBisCO. Further research could better investigate the biochemical relationship between changes in  $N_{leaf}$  and changes of N concentrations in RuBisCO to confirm the hypothesis raised in the present study. Nevertheless, the present study adopted extreme N:P ratios, particularly unrealistic in the high P solution. Therefore, further studies could select more reasonable nutrient ratios to better represent plant responses in natural ecosystems suffering soil P limitation. Moreover, the present study did not consider the ongoing anthropogenic alteration of the N cycle, although N has a strong influence on NPP and plants

photosynthetic and physiological responses and it is likely that its biologically available form has been increasing since the industrial revolution because of human activity. As such, future studies could investigate plant responses to rising  $Ca$  while simultaneously decreasing  $P_s$  and increasing  $N_s$ .

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## Appendix A: Difference in responses between *H.lanatus* and *S.dulcamara*

**Table 1A.** Differences in  $N_{leaf}$ ,  $P_{leaf}$ ,  $V_{cmax}$  and  $J_{max}$  responses between *H.lanatus* and *S.dulcamara* to the combined treatment of rising  $Ca$  and low  $P_s$ .

Traits	Difference between <i>H.lanatus</i> and <i>S.dulcamara</i>
$N_{leaf}$	*
$P_{leaf}$	n.S
$V_{cmax}$	***
$J_{max}$	***

ns = not significant + =  $P \leq 0,10$  \* =  $P \leq 0,05$  \*\* =  $P \leq 0,01$  \*\*\* =  $P \leq 0,001$

## Appendix B: Results per individual

**Table 1B.** List of the results per individuals for all the traits analyzed in the study in the three species (*H.lanatus*, *S.dulcamara* and *P.miliaceum*). Units are the following: For  $B_t$  (g), for SLA and LAR ( $\frac{cm^2}{g}$ ), for  $N_{leaf}$  and  $P_{leaf}$  ( $\frac{mg}{Kg}$ ), for  $V_{cmax}$  and  $J_{max}$  ( $\mu mol\ m^{-2}\ s^{-1}$ ).  $B_t$ ,  $B_a:B_b$  and LMR of *P.miliaceum* are often nine even if only eight out of them were selected.

Species	Treatment ( $Ca_{P_s}$ )	Individual	$B_t$	$B_a:B_b$	LMR	SLA	LAR	$N_{leaf}$	$P_{leaf}$	$V_{cmax}$	$J_{max}$
<i>H.lanatus</i>	LowCa_lowP <sub>s</sub>	green1l	3,62	0,88	0,47	181	84	-	-	39	79
<i>H.lanatus</i>	LowCa_lowP <sub>s</sub>	green5l	3,91	1,03	0,51	204	104	28024	853	28	55
<i>H.lanatus</i>	LowCa_lowP <sub>s</sub>	green9l	3,89	0,68	0,41	161	65	-	-	39	57
<i>H.lanatus</i>	LowCa_lowP <sub>s</sub>	grey10l	-	-	-	-	-	-	-	49	66
<i>H.lanatus</i>	LowCa_lowP <sub>s</sub>	grey2l	-	-	-	-	-	-	-	53	80
<i>H.lanatus</i>	LowCa_lowP <sub>s</sub>	grey4l	2,62	0,95	0,49	201	98	-	-	59	80
<i>H.lanatus</i>	LowCa_lowP <sub>s</sub>	grey6l	4,95	1,02	0,51	220	111	34058	658	48	71
<i>H.lanatus</i>	LowCa_lowP <sub>s</sub>	green3l	4,17	0,85	0,46	193	89	36058	1439	-	-
<i>H.lanatus</i>	LowCa_lowP <sub>s</sub>	green11l	3,67	0,84	0,46	224	102	33952	867	-	-
<i>H.lanatus</i>	LowCa_lowP <sub>s</sub>	green7l	3,18	0,68	0,40	189	76	36089	775	-	-
<i>H.lanatus</i>	LowCa_highP <sub>s</sub>	green12l	3,99	1,17	0,54	Missing	Missing	19890	10824	59	91
<i>H.lanatus</i>	LowCa_highP <sub>s</sub>	green4l	4,15	1,44	0,59	Missing	Missing	-	-	57	83
<i>H.lanatus</i>	LowCa_highP <sub>s</sub>	green6l	-	-	-	-	-	-	-	42	89
<i>H.lanatus</i>	LowCa_highP <sub>s</sub>	green8l	3,94	1,89	0,65	Missing	Missing	19091	7777	51	81
<i>H.lanatus</i>	LowCa_highP <sub>s</sub>	grey1l	-	-	-	-	-	-	-	49	92
<i>H.lanatus</i>	LowCa_highP <sub>s</sub>	grey3l	3,88	1,46	0,59	Missing	Missing	23041	9373	43	83
<i>H.lanatus</i>	LowCa_highP <sub>s</sub>	grey5l	-	-	-	-	-	-	-	56	78
<i>H.lanatus</i>	LowCa_highP <sub>s</sub>	#9 gray	3,94	1,55	0,61	Missing	Missing	-	-	-	-
<i>H.lanatus</i>	LowCa_highP <sub>s</sub>	#2 green	5,17	1,07	0,52	Missing	Missing	-	-	-	-
<i>H.lanatus</i>	LowCa_highP <sub>s</sub>	#10 green	4,70	1,49	0,60	Missing	Missing	24618	7442	-	-
<i>H.lanatus</i>	LowCa_highP <sub>s</sub>	#11 gray	3,47	1,85	0,65	Missing	Missing	28401	9128	-	-
<i>H.lanatus</i>	AmbientCa_lowP <sub>s</sub>	green10a	-	-	-	-	-	-	-	11	29

<i>H.lanatus</i>	AmbientCa_lowP <sub>s</sub>	green12a	10,02	0,50	0,33	171	57	13418	344	29	60
<i>H.lanatus</i>	AmbientCa_lowP <sub>s</sub>	green2a	6,65	0,65	0,39	200	79	16440	302	23	53
<i>H.lanatus</i>	AmbientCa_lowP <sub>s</sub>	green4a	5,21	0,48	0,33	227	74	30912	746	16	40
<i>H.lanatus</i>	AmbientCa_lowP <sub>s</sub>	grey12a	-	-	-	-	-	-	-	23	38
<i>H.lanatus</i>	AmbientCa_lowP <sub>s</sub>	grey2a	7,13	0,37	0,27	178	48	-	-	17	43
<i>H.lanatus</i>	AmbientCa_lowP <sub>s</sub>	grey6a	-	-	-	-	-	-	-	22	50
<i>H.lanatus</i>	AmbientCa_lowP <sub>s</sub>	#8 gray	4,43	0,59	0,37	211	78	31443	542	-	-
<i>H.lanatus</i>	AmbientCa_lowP <sub>s</sub>	#4 gray	5,21	0,43	0,30	199	59	32220	446	-	-
<i>H.lanatus</i>	AmbientCa_lowP <sub>s</sub>	#6 green	7,80	0,48	0,32	144	47	-	-	-	-
<i>H.lanatus</i>	AmbientCa_lowP <sub>s</sub>	#8 green	7,02	0,71	0,42	170	70	-	-	-	-
<i>H.lanatus</i>	AmbientCa_highP <sub>s</sub>	green11a	12,60	0,69	0,41	182	75	10711	3653	37	71
<i>H.lanatus</i>	AmbientCa_highP <sub>s</sub>	green3a	12,11	0,61	0,38	180	69	-	-	35	79
<i>H.lanatus</i>	AmbientCa_highP <sub>s</sub>	green7a	12,21	0,44	0,30	219	67	12507	4260	34	77
<i>H.lanatus</i>	AmbientCa_highP <sub>s</sub>	green9a	10,97	0,46	0,32	289	92	22352	4946	36	67
<i>H.lanatus</i>	AmbientCa_highP <sub>s</sub>	grey1a	12,23	0,48	0,32	177	57	-	-	26	92
<i>H.lanatus</i>	AmbientCa_highP <sub>s</sub>	grey3a	-	-	-	-	-	-	-	42	77
<i>H.lanatus</i>	AmbientCa_highP <sub>s</sub>	grey7a	11,82	0,49	0,33	186	62	-	-	40	82
<i>H.lanatus</i>	AmbientCa_highP <sub>s</sub>	#5 gray	12,31	0,42	0,30	180	54	13737	7454	-	-
<i>H.lanatus</i>	AmbientCa_highP <sub>s</sub>	#5 green	12,62	0,45	0,31	194	60	18976	5958	-	-
<i>H.lanatus</i>	HighCa_lowP <sub>s</sub>	green1h	-	-	-	-	-	-	-	10	27
<i>H.lanatus</i>	HighCa_lowP <sub>s</sub>	green3h	7,51	0,70	0,41	195	80	17971	320	19	46
<i>H.lanatus</i>	HighCa_lowP <sub>s</sub>	green7h	7,31	0,73	0,42	194	82	16466	884	24	49
<i>H.lanatus</i>	HighCa_lowP <sub>s</sub>	grey10h	5,56	0,41	0,29	278	81	33967	622	18	32
<i>H.lanatus</i>	HighCa_lowP <sub>s</sub>	grey12h	-	-	-	-	-	-	-	16	38
<i>H.lanatus</i>	HighCa_lowP <sub>s</sub>	grey6h	4,60	0,67	0,40	235	94	28222	425	14	36
<i>H.lanatus</i>	HighCa_lowP <sub>s</sub>	grey8h	3,81	0,50	0,33	263	87	-	-	12	31
<i>H.lanatus</i>	HighCa_lowP <sub>s</sub>	#4 gray	5,06	0,60	0,38	214	80	19126	456	-	-
<i>H.lanatus</i>	HighCa_lowP <sub>s</sub>	#5 green	5,50	0,57	0,36	267	97	-	-	-	-
<i>H.lanatus</i>	HighCa_lowP <sub>s</sub>	#9 green	5,72	0,50	0,33	277	92	-	-	-	-
<i>H.lanatus</i>	HighCa_highP <sub>s</sub>	green10h	-	-	-	-	-	-	-	29	67
<i>H.lanatus</i>	HighCa_highP <sub>s</sub>	green2h	14,04	0,50	0,34	159	53	-	-	32	65
<i>H.lanatus</i>	HighCa_highP <sub>s</sub>	green4h	13,21	0,52	0,34	279	95	9998	4043	29	72
<i>H.lanatus</i>	HighCa_highP <sub>s</sub>	green6h	10,60	0,53	0,35	171	59	8154	3817	21	37
<i>H.lanatus</i>	HighCa_highP <sub>s</sub>	grey11h	-	-	-	-	-	-	-	32	65
<i>H.lanatus</i>	HighCa_highP <sub>s</sub>	grey1h	12,32	0,64	0,39	218	85	8176	2590	32	67
<i>H.lanatus</i>	HighCa_highP <sub>s</sub>	grey7h	12,37	0,47	0,32	325	104	-	-	25	61
<i>H.lanatus</i>	HighCa_highP <sub>s</sub>	#5 green	14,27	0,40	0,29	176	50	9931	6473	-	-
<i>H.lanatus</i>	HighCa_highP <sub>s</sub>	# 8 green	9,78	0,78	0,44	282	123	-	-	-	-

<i>H.lanatus</i>	HighCa_highP <sub>s</sub>	#3 gray	12,24	0,86	0,46	186	86	12462	3061	-	-
<i>S.dulcamara</i>	LowCa_lowP <sub>s</sub>	purple12l	-	-	-	-	-	-	-	62	104
<i>S.dulcamara</i>	LowCa_lowP <sub>s</sub>	purple2l	0,97	1,16	0,03	282	9	51411	2105	61	96
<i>S.dulcamara</i>	LowCa_lowP <sub>s</sub>	purple9l	0,64	0,93	0,04	377	16	58372	2581	61	90
<i>S.dulcamara</i>	LowCa_lowP <sub>s</sub>	white3l	-	-	-	-	-	-	-	63	90
<i>S.dulcamara</i>	LowCa_lowP <sub>s</sub>	white6l	0,30	0,80	0,13	388	51	58930	7472	93	103
<i>S.dulcamara</i>	LowCa_lowP <sub>s</sub>	white8l	0,36	1,43	0,08	367	29	51957	2715	67	99
<i>S.dulcamara</i>	LowCa_lowP <sub>s</sub>	#8 purple	0,70	1,32	0,02	304	5	-	-	-	-
<i>S.dulcamara</i>	LowCa_lowP <sub>s</sub>	#10 white	0,67	1,44	0,01	298	4	-	-	-	-
<i>S.dulcamara</i>	LowCa_lowP <sub>s</sub>	#11 white	0,34	0,61	0,08	412	31	-	-	-	-
<i>S.dulcamara</i>	LowCa_lowP <sub>s</sub>	#1 white	0,64	1,56	0,05	221	11	47384	2417	-	-
<i>S.dulcamara</i>	LowCa_highP <sub>s</sub>	purple1l	1,66	1,29	0,03	350	12	-	-	100	125
<i>S.dulcamara</i>	LowCa_highP <sub>s</sub>	purple4l	-	-	-	-	-	-	-	94	113
<i>S.dulcamara</i>	LowCa_highP <sub>s</sub>	purple7l	-	-	-	-	-	-	-	89	134
<i>S.dulcamara</i>	LowCa_highP <sub>s</sub>	white2l	2,00	1,29	0,06	344	21	56643	5851	92	116
<i>S.dulcamara</i>	LowCa_highP <sub>s</sub>	white4l	-	-	-	-	-	-	-	43	82
<i>S.dulcamara</i>	LowCa_highP <sub>s</sub>	white5l	1,55	1,23	0,04	208	8	-	-	91	121
<i>S.dulcamara</i>	LowCa_highP <sub>s</sub>	#9white	2,61	1,21	0,03	328	9	-	-	-	-
<i>S.dulcamara</i>	LowCa_highP <sub>s</sub>	#7 white	1,80	1,53	0,04	376	13	48448	7785	-	-
<i>S.dulcamara</i>	LowCa_highP <sub>s</sub>	#10 purple	2,76	1,92	0,12	410	50	31669	5625	-	-
<i>S.dulcamara</i>	LowCa_highP <sub>s</sub>	#3 purple	1,86	1,17	0,03	354	12	48327	7680	-	-
<i>S.dulcamara</i>	LowCa_highP <sub>s</sub>	#12 white	2,14	1,27	0,03	338	10	49499	9797	-	-
<i>S.dulcamara</i>	AmbientCa_lowP <sub>s</sub>	purple10a	1,89	0,98	0,09	128	11	-	-	30	59
<i>S.dulcamara</i>	AmbientCa_lowP <sub>s</sub>	purple11a	2,35	1,31	0,06	183	11	20128	781	48	79
<i>S.dulcamara</i>	AmbientCa_lowP <sub>s</sub>	purple2a	1,92	1,11	0,07	200	14	19926	792	34	63
<i>S.dulcamara</i>	AmbientCa_lowP <sub>s</sub>	purple8a	2,00	1,00	0,12	155	18	12922	674	22	52
<i>S.dulcamara</i>	AmbientCa_lowP <sub>s</sub>	white12a	2,46	1,09	0,14	208	30	15510	820	34	64
<i>S.dulcamara</i>	AmbientCa_lowP <sub>s</sub>	white8a	-	-	-	-	-	-	-	31	65
<i>S.dulcamara</i>	AmbientCa_lowP <sub>s</sub>	#4 purple	0,72	1,34	0,15	259	39	-	-	-	-
<i>S.dulcamara</i>	AmbientCa_lowP <sub>s</sub>	#11 white	1,21	1,42	0,07	158	11	-	-	-	-
<i>S.dulcamara</i>	AmbientCa_lowP <sub>s</sub>	#6 purple	1,78	1,03	0,06	220	13	18843	1001	-	-
<i>S.dulcamara</i>	AmbientCa_highP <sub>s</sub>	purple12a	4,50	1,63	0,16	180	29	-	-	35	74
<i>S.dulcamara</i>	AmbientCa_highP <sub>s</sub>	purple1a	-	-	-	-	-	-	-	44	87
<i>S.dulcamara</i>	AmbientCa_highP <sub>s</sub>	white1a	3,80	1,76	0,08	229	17	-	-	44	83
<i>S.dulcamara</i>	AmbientCa_highP <sub>s</sub>	white3a	-	-	-	-	-	-	-	39	78
<i>S.dulcamara</i>	AmbientCa_highP <sub>s</sub>	white5a	6,17	1,58	0,18	231	41	7867	7777	35	63
<i>S.dulcamara</i>	AmbientCa_highP <sub>s</sub>	white9a	-	-	-	-	-	-	-	33	67
<i>S.dulcamara</i>	AmbientCa_highP <sub>s</sub>	#7 white	4,25	1,82	0,13	192	26	-	-	-	-
<i>S.dulcamara</i>	AmbientCa_highP <sub>s</sub>	#5 purple	3,64	1,16	0,07	174	12	10602	2151	-	-



<i>S.dulcamara</i>	AmbientCa_highP <sub>s</sub>	#7 purple	4,69	1,58	0,13	247	32	8258	2906	-	-
<i>S.dulcamara</i>	AmbientCa_highP <sub>s</sub>	#3 purple	3,57	1,11	0,08	222	17	10631	823	-	-
<i>S.dulcamara</i>	AmbientCa_highP <sub>s</sub>	#10 white	3,21	1,83	0,13	224	29	13517	2447	-	-
<i>S.dulcamara</i>	HighCa_lowP <sub>s</sub>	purple12h	-	-	-	-	-	-	-	21	52
<i>S.dulcamara</i>	HighCa_lowP <sub>s</sub>	purple12h1	-	-	-	-	-	-	-	25	59
<i>S.dulcamara</i>	HighCa_lowP <sub>s</sub>	purple1h	1,95	1,38	0,04	156	7	15988	822	9	23
<i>S.dulcamara</i>	HighCa_lowP <sub>s</sub>	purple5h	2,00	1,80	0,10	196	20	-	-	24	49
<i>S.dulcamara</i>	HighCa_lowP <sub>s</sub>	purple9h	2,55	1,54	0,09	209	20	14377	1158	30	58
<i>S.dulcamara</i>	HighCa_lowP <sub>s</sub>	white1h	2,58	1,27	0,12	175	21	-	-	24	49
<i>S.dulcamara</i>	HighCa_lowP <sub>s</sub>	white5h	0,85	0,86	0,20	138	27	13267	926	31	73
<i>S.dulcamara</i>	HighCa_lowP <sub>s</sub>	#3 purple	1,49	1,55	0,07	195	14	-	-	-	-
<i>S.dulcamara</i>	HighCa_lowP <sub>s</sub>	#7 purple	2,06	1,27	0,06	196	12	18194	682	-	-
<i>S.dulcamara</i>	HighCa_lowP <sub>s</sub>	#7 white	1,47	1,11	0,15	183	27	13939	615	-	-
<i>S.dulcamara</i>	HighCa_highP <sub>s</sub>	purple10h	-	-	-	-	-	-	-	32	58
<i>S.dulcamara</i>	HighCa_highP <sub>s</sub>	purple6h	-	-	-	-	-	-	-	20	38
<i>S.dulcamara</i>	HighCa_highP <sub>s</sub>	purple8h	-	-	-	-	-	-	-	20	44
<i>S.dulcamara</i>	HighCa_highP <sub>s</sub>	white12h	5,95	1,61	0,14	167	23	7011	3138	30	61
<i>S.dulcamara</i>	HighCa_highP <sub>s</sub>	white6h	6,03	2,21	0,10	195	19	-	-	29	71
<i>S.dulcamara</i>	HighCa_highP <sub>s</sub>	white8h	7,25	1,78	0,17	166	29	7076	2295	23	56
<i>S.dulcamara</i>	HighCa_highP <sub>s</sub>	white8h1	-	-	-	-	-	-	-	27	60
<i>S.dulcamara</i>	HighCa_highP <sub>s</sub>	#2 purple	4,57	2,14	0,11	285	30	-	-	-	-
<i>S.dulcamara</i>	HighCa_highP <sub>s</sub>	#4 white	5,31	1,61	0,15	162	24	7264	2907	-	-
<i>S.dulcamara</i>	HighCa_highP <sub>s</sub>	#2 white	5,41	1,67	0,10	214	21	-	-	-	-
<i>S.dulcamara</i>	HighCa_highP <sub>s</sub>	#11 purple	4,38	1,78	0,07	273	20	8634	3219	-	-
<i>S.dulcamara</i>	HighCa_highP <sub>s</sub>	#11 white	6,35	2,09	0,13	170	22	6872	2472	-	-
<i>P.miliaceum</i>	LowCa_lowP <sub>s</sub>	1	1,50	4,71	7,04	293	2064	15270	462	-	-
<i>P.miliaceum</i>	LowCa_lowP <sub>s</sub>	2	1,81	3,58	5,12	264	1352	23820	835	-	-
<i>P.miliaceum</i>	LowCa_lowP <sub>s</sub>	3	2,05	5,29	3,20	188	603	20230	1454	-	-
<i>P.miliaceum</i>	LowCa_lowP <sub>s</sub>	4	1,45	3,83	2,36	-	-	-	-	-	-
<i>P.miliaceum</i>	LowCa_lowP <sub>s</sub>	5	1,47	3,33	4,35	260	1131	14760	471	-	-
<i>P.miliaceum</i>	LowCa_lowP <sub>s</sub>	6	1,53	4,59	3,70	215	793	33140	2022	-	-
<i>P.miliaceum</i>	LowCa_lowP <sub>s</sub>	7	1,44	6,21	2,91	260	756	-	-	-	-
<i>P.miliaceum</i>	LowCa_lowP <sub>s</sub>	8	1,46	20,81	1,91	-	-	-	-	-	-
<i>P.miliaceum</i>	LowCa_lowP <sub>s</sub>	9	1,71	3,64	2,58	-	-	-	-	-	-
<i>P.miliaceum</i>	LowCa_highP <sub>s</sub>	1	1,61	3,71	3,31	211	698	40330	23954	-	-
<i>P.miliaceum</i>	LowCa_highP <sub>s</sub>	2	2,06	3,67	3,78	167	630	47440	22046	-	-
<i>P.miliaceum</i>	LowCa_highP <sub>s</sub>	3	1,56	3,69	4,70	332	1559	-	-	-	-
<i>P.miliaceum</i>	LowCa_highP <sub>s</sub>	4	1,35	3,67	3,61	210	757	51910	28001	-	-
<i>P.miliaceum</i>	LowCa_highP <sub>s</sub>	5	1,46	4,26	2,37	-	-	-	-	-	-

<i>P.miliaceum</i>	LowCa_highP <sub>s</sub>	6	1,52	3,14	2,36	-	-	-	-	-	-
<i>P.miliaceum</i>	LowCa_highP <sub>s</sub>	7	1,35	2,19	2,24	-	-	-	-	-	-
<i>P.miliaceum</i>	LowCa_highP <sub>s</sub>	8	1,52	2,01	4,02	158	633	42240	27966	-	-
<i>P.miliaceum</i>	LowCa_highP <sub>s</sub>	9	1,73	1,36	4,74	219	1036	42920	33113	-	-
<i>P.miliaceum</i>	AmbientCa_lowP <sub>s</sub>	1	2,26	4,17	3,14	211	663	20760	1024	-	-
<i>P.miliaceum</i>	AmbientCa_lowP <sub>s</sub>	2	2,16	8,94	3,01	-	-	-	-	-	-
<i>P.miliaceum</i>	AmbientCa_lowP <sub>s</sub>	3	1,70	6,40	2,58	-	-	-	-	-	-
<i>P.miliaceum</i>	AmbientCa_lowP <sub>s</sub>	4	2,34	7,29	3,34	248	828	24210	683	-	-
<i>P.miliaceum</i>	AmbientCa_lowP <sub>s</sub>	5	2,43	5,48	3,54	239	845	15590	543	-	-
<i>P.miliaceum</i>	AmbientCa_lowP <sub>s</sub>	6	1,86	10,61	3,06	239	729	-	-	-	-
<i>P.miliaceum</i>	AmbientCa_lowP <sub>s</sub>	7	1,75	7,15	3,14	239	750	20403	753	-	-
<i>P.miliaceum</i>	AmbientCa_lowP <sub>s</sub>	8	2,06	6,66	3,17	244	772	20980	942	-	-
<i>P.miliaceum</i>	AmbientCa_highP <sub>s</sub>	1	4,04	2,46	4,81	-	-	-	-	-	-
<i>P.miliaceum</i>	AmbientCa_highP <sub>s</sub>	2	3,99	5,67	5,27	261	1374	12700	14953	-	-
<i>P.miliaceum</i>	AmbientCa_highP <sub>s</sub>	3	3,28	4,25	4,78	212	1012	16680	15349	-	-
<i>P.miliaceum</i>	AmbientCa_highP <sub>s</sub>	4	3,22	4,86	4,82	225	1087	18360	19580	-	-
<i>P.miliaceum</i>	AmbientCa_highP <sub>s</sub>	5	3,19	4,60	5,69	260	1481	15510	15701	-	-
<i>P.miliaceum</i>	AmbientCa_highP <sub>s</sub>	6	3,14	4,71	3,89	-	-	-	-	-	-
<i>P.miliaceum</i>	AmbientCa_highP <sub>s</sub>	7	2,99	5,09	3,81	-	-	-	-	-	-
<i>P.miliaceum</i>	AmbientCa_highP <sub>s</sub>	8	4,15	2,64	6,37	229	1457	13760	19756	-	-
<i>P.miliaceum</i>	AmbientCa_highP <sub>s</sub>	9	4,06	3,81	5,13	138	705	-	-	-	-
<i>P.miliaceum</i>	HighCa_lowP <sub>s</sub>	1	2,76	4,19	4,94	297	1470	-	-	-	-
<i>P.miliaceum</i>	HighCa_lowP <sub>s</sub>	2	2,48	3,60	3,74	301	1128	23890	653	-	-
<i>P.miliaceum</i>	HighCa_lowP <sub>s</sub>	3	2,50	5,54	3,31	-	-	-	-	-	-
<i>P.miliaceum</i>	HighCa_lowP <sub>s</sub>	4	2,86	3,02	4,14	247	1025	22620	570	-	-
<i>P.miliaceum</i>	HighCa_lowP <sub>s</sub>	5	2,72	4,04	3,83	215	823	27010	972	-	-
<i>P.miliaceum</i>	HighCa_lowP <sub>s</sub>	6	2,64	4,36	4,36	242	1056	20890	503	-	-
<i>P.miliaceum</i>	HighCa_lowP <sub>s</sub>	7	2,87	6,45	3,68	-	-	-	-	-	-
<i>P.miliaceum</i>	HighCa_lowP <sub>s</sub>	8	2,72	3,88	3,55	-	-	-	-	-	-
<i>P.miliaceum</i>	HighCa_lowP <sub>s</sub>	9	2,34	5,10	4,50	294	1324	22420	354	-	-
<i>P.miliaceum</i>	HighCa_highP <sub>s</sub>	1	4,16	3,59	6,16	228	1406	-	-	-	-
<i>P.miliaceum</i>	HighCa_highP <sub>s</sub>	2	3,75	3,66	5,39	260	1401	11700	13640	-	-
<i>P.miliaceum</i>	HighCa_highP <sub>s</sub>	3	3,03	5,69	3,75	-	-	-	-	-	-
<i>P.miliaceum</i>	HighCa_highP <sub>s</sub>	4	4,24	3,85	6,07	205	1242	12890	15408	-	-
<i>P.miliaceum</i>	HighCa_highP <sub>s</sub>	5	3,42	7,03	4,15	-	-	-	-	-	-
<i>P.miliaceum</i>	HighCa_highP <sub>s</sub>	6	3,58	2,66	4,26	-	-	-	-	-	-
<i>P.miliaceum</i>	HighCa_highP <sub>s</sub>	7	4,18	5,17	7,34	231	1696	15550	13465	-	-
<i>P.miliaceum</i>	HighCa_highP <sub>s</sub>	8	4,30	3,66	6,43	177	1141	22160	10795	-	-
<i>P.miliaceum</i>	HighCa_highP <sub>s</sub>	10	3,86	4,39	7,18	221	1586	19260	10898	-	-

