Master's Thesis

Sustainable Development

The impact of the photosynthetic traits' downregulation on the leaf nutrient composition

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Preface

This thesis was written as part of the Sustainable Development master program at Utrecht University, in the Environmental Change and Ecosystems track. The research focused on the relationship between the photosynthesis traits and macro- and micronutrients, to increase the understanding of the impact of the abiotic changing factor atmospheric CO_2 on the plant's nutrient content. During the thesis, I did an internship at the nutrition company DSM. The team I worked in was engaged in global malnutrition projects, together with WFP and UNICEF and did research on the impact of climate change on nutrient deficiencies and how certain innovations can tackle the nutrition crisis. I want to thank my internship supervisor Florentine Oberman for the excellent supervision. After the internship I worked in the Utrecht University' Environmental Laboratory. Throughout the entire process, I have been supervised by Dr. Hugo de Boer and I would like to sincerely thank him for his advice, support and feedback. I would also like to thank the lab supervisors Thom Claessen and Desmond Eefting for their assistance. Furthermore I would like to thank the PhD students Jan Lankhorst and Astrid Odé for the great collaboration with the lab work. Finally, I would like to thank Dr. Karin Rebel for being the second reader of my thesis and for inspiring me to sign up for the bachelor program Global Sustainability Science, which has been the start of my enthusiasm for environmental sciences.

Abstract

The atmospheric CO_2 concentration determines to a large extent how plants perform their photosynthesis. The photosynthetic parameters, V_{cmax} and J_{max}, explain respectively the maximum rate of RuBisCo carboxylase activity and the maximum rate of photosynthetic electron transport. Prior research evidenced a positive relation between these photosynthetic traits and leaf nitrogen (Smith et al., 2019). This research aims to analyse the relationship between the photosynthetic parameters, nitrogen and phosphorus and the micronutrient content (zinc, iron and calcium) of four plant species. The plant material has been derived from a field study in the Ebro basin (north-eastern Spain). The photosynthetic parameters have been measured by using a leaf gas exchange machine. Input from the experience during the internship at the nutrition company DSM has been used to determine which micronutrients (iron, zinc and calcium) should be focused on to increase the relevance of this research. For the elemental analysis of the leaf material, two machines have been used: the CN-analyser and the Picofox TXRF spectrometer. The created dataset contains data from 34 samples, including five groups (comprising four species: alfalfa, almond, grape, and apple; the latter including irrigated and non-irrigated samples), analysed on photosynthetic activity and elemental content. The results of the research have been derived by using analysis of (co)variance (ANOVA and ANCOVA) statistical tests and by analysing the variance for the absolute values for micronutrients compared to the variance with the ratios with nitrogen (N) and phosphorus (P). The results of the F-test indicate a decrease in variance with the ratios with N and P compared to the absolute values of micronutrients, which gives insights in the stoichiometry of the leaf. The ANOVA tests reveal a significant difference in nutrient content per plant species. When performing the ANCOVA tests, it became clear that a significant correlation exists between J_{max} and V_{cmax} and the P concentration. Also, the P concentration is a significant determinant for the absolute zinc (Zn) concentration and the Zn:N and Zn:P ratio in the plant, this is a positive correlation. To conclude, the results of this research indicate that an increase in CO_2 leads to a downregulation of the photosynthetic traits, resulting in a lower concentration of leaf P, and a significantly lower concentration of Zn in the leaf. Further research is needed to perform this analysis on a larger sample size and to analyse other plant material besides the leaf, such as fruits and grains.

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Abbreviations

C	Carbon
Ca	Calcium
CO_2	Carbon-dioxide
ET	Electron Transport
Fe	Iron
IRTA	Institute of Agrifood Research and Technology
J_{max}	Maximum electron transport
LMA	Leaf Mass per Area
N	Nitrogen
P	Phosphorus
PAR	Photosynthetic active radiation
RCP	Representative Concentration Pathways
RuBisCo	Ribulose-1,5-bisphosphate carboxylase-oxygenase
SLA	Specific Leaf Area
V_{cmax}	Maximum velocity rate of carboxylation
Zn	Zinc

Introduction

The Representative Concentration Pathways (RCPs) projected a doubling of atmospheric CO_2 production from 380 to 700 ca. μmol C mol^{-1} (IPCC, 2007) and a mean surface temperature increase of 2.6 - 4.8 °C by 2100 when following current pathways of carbon emissions (Qin et al., 2014; Leisner, 2020). The potential doubling of atmospheric CO_2 concentrations may dramatically impair the performance of photosynthesis in plants (Vandegeer et al., 2012). In order to estimate the consequences of increasing CO_2 concentrations on terrestrial crops, it is necessary to analyse the photosynthetic engineering traits. Several studies have revealed the contribution of these parameters - the maximum carboxylation (V_{cmax}) and the maximum electron transport (ET) rate (J_{max}) - to yield enhancement (von Caemmerer & Evans, 2010; Evans, 2013; Long et al., 2015). Elevated CO_2 levels modify guard cells, which reduces stomatal aperture and the quantity of stomata per leaf, resulting in a reduction of stomatal conductance (Farquhar & Sharkey, 1982; Keenan et al., 2013). A decreased opening of the stoma may limit the process of evapotranspiration, which enhances heat stress of the plant (Thomas et al., 2016). This in combination with the expected droughts and elevated temperatures can affect photosynthesis, the plants health and consequently reduce the productivity of the crop. The decrease in stomatal conductance results in a downregulation of the photosynthetic traits J_{max} and V_{cmax} (Liu et al., 2014). Prior research showed evidence of the correlation between these photosynthetic traits and the leaf nitrogen (N) and phosphorus (P) concentration (Walker et al., 2014). Theoretical and experimental evidence suggests that J_{max} and V_{cmax} scale with leaf nitrogen (N) via the significant amount of N invested in the ribulose 1-5-bisphosphate oxygenase-carboxylase (RuBisCo) protein, and that phosphorus (P) availability affects many aspects of plant physiology essential to photosynthesis, such as membrane solubility and ATP and NADPH production (Marschner 1995; Taiz & Zeiger 2010)

Anthropogenic CO_2 emissions threaten human nutrition in two ways. The first is the disruption of the global climate system with all the associated impacts on food production (droughts, floods, humidity changes) (Myers et al., 2017). Secondly, the direct altering of the nutrient profile of staple food crops. Carbon, the major constituent of terrestrial vascular plants is obtained from atmospheric CO_2, all other chemical elements that are crucial for a plant's existence are derived from the soil. The way these concentrations of nutrients change is not in unison with the increasing concentrations of CO_2 (Loladze, 2002). Consequently, modern plants are experiencing a global elemental imbalance compared to pre-industrial times which could result in nutrient deficiencies for humans (Loladze, 2002). Moreover, elevated CO_2 concentrations are altering the growth rate of plants by influencing the plants productivity. This additionally influences the ability of the world to facilitate adequate food production for the growing population (Leisner, 2020). This effect on the nutrient content of plants is likely to reduce the dietary supply of nutrients for many populations and increase the prevalence of global nutritional insufficiency (Smith & Meyers, 2018).

According to the United Nations' Food and Agriculture Organisation (FAO, 2012), a food production increase of approximately 70% is needed to feed the world's population which is estimated at 9 billion people in 2050 (Tester & Langridge, 2010; Hussain et al. 2020a,b). Several essential nutrients are lacking in modern crops, resulting in the 'hidden hunger' problem; the suffering from micronutrient deficiencies (Graham et al., 2001; WHO, 2006). To meet the challenge of this huge enhancement of the global food demand, improvement of crop production is required and nutrient losses must be avoided. A thorough understanding of the impact of climate change, as a result of atmospheric CO_2 increase and the changes made in the stoichiometry of plants is essential to ensure global food security (Sterner & Elser, 2017; Hussain et al., 2021). Ecological stoichiometry is the study of the balance of energy and multiple chemical elements in living systems, and how this balance of plant-internal elements is influenced by the biotic and abiotic environment (Elser et al., 2000; Knecht & Göransson, 2004; Ladanai et al., 2010). Understanding the stoichiometry of plants is necessary to calculate the influences of biotic and abiotic changes on the plant's composition. Ågren & Weih (2020) state that most stoichiometry research has been done on the elements carbon, nitrogen and phosphorus and that more research needs to be done on the stoichiometry with other elements. This research will focus on the stoichiometry between macro and micronutrients in plants, and how this stoichiometry is related to the photosynthetic traits V_{cmax} and J_{max}. This way, the impact of CO_2 on the micronutrient content of plants can be researched.

Theory and Concepts

2.1 Photosynthesis performance

Photosynthesis is the basis of plant growth, it is a biological process whereby light energy is captured and stored by a living organism, and converted from light energy to biochemical energy which is used to drive energy-requiring cellular processes (Evans, 2013). The process occurs according to the Calvin Cycle, which is a series of biochemical redox reactions that take place in the stroma of the chloroplasts (Bassham et al., 1950; Blankenship, 2021). The sun's output radiation with a wavelength between 400 and 700 nm is defined as the Photosynthetically Active Radiation (PAR); this light is used in the most familiar photosynthesizing organisms: the chlorophyll *a*-containing organisms. Plants can carry out photosynthesis in three different ways: C_3, C_4 and CAM photosynthesis. The majority of plant species use C_3 photosynthesis (89% of plants), in which the first carbon compound produced contains three carbon atoms (Carvajal, 2010). However, some plants have evolved an additional form of photosynthesis, C_4 (which produces a four-carbon compound) to help reduce these losses in dry environments (Blankenship, 2021). CAM type of photosynthesis is an adaptation to low water availability and occurs in orchids and succulent plant species from arid regions, the stomata in the leaves are closed during daylight to lessen evapotranspiration and open at night to absorb CO_2 (Wang et al., 2008). The most important difference is how the CO_2 fixation pathway occurs (Evans, 2013). All C_3 plants use RuBisCo - a carboxylase enzyme that also catalyses a reaction with oxygen that diminishes the overall efficiency of photosynthesis (Evans, 2013). The maximum catalytic activity of the RuBisCo enzyme is defined as V_{cmax} (Leuning, 1997). In this process, 3-phosphoglycerate (3GPA) is created. V_{cmax} and the maximum rate of photosynthetic electron transport (J_{max}) define plant photosynthetic capacity at the leaf level, and are referred to as the photosynthetic traits (Fan et al., 2011). This research will focus only on species using C_3 photosynthesis, because most plants perform C_3 photosynthesis and because response curves to derive V_{cmax} and J_{max} will be used that cannot derive the photosynthesis traits with this model for C_4 or CAM species.

2.2 Influence of CO_2 on photosynthesis

A plant's elemental chemical composition reflects a balance between carbon, retrieved from atmospheric CO_2, and the remaining nutrients, which come from the soil (Loladze, 2002). Projected increases in atmospheric CO_2 can result in an ionomic imbalance for most plant species, where carbon increases disproportionately compared to soil-based nutrients (Ainsworth & Long, 2005; Bernacchi et al., 2005; Bernacchi et al., 2006; Ainsworth & Rogers, 2007; Taub et al., 2008; Loladze, 2014; Müller et al., 2014; Myers et al., 2014). Higher levels of CO_2 generally boost the net photosynthetic rate (A_n) of plants, namely "CO_2 fertilisation effect", especially for C_3 plants. This can be explained by the

ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCo) of C_3 plants being CO_2-saturated with increased atmospheric CO_2 concentration (Curtis & Wang, 1998; Lee et al., 2001; Morgan et al., 2007; Yu et al., 2012; Singh & Reddy, 2016). This eventually increases the C_3 plant's photosynthetic performance (Zhu et al., 2007; Leakey et al., 2009). A consequence of an increased photosynthetic performance per unit leaf area may be an increased plant growth (Hussein et al., 2021). Also, prior research showed evidence that the rate of photosynthesis is relatively higher in small leaves compared to larger leaves (Bronstein et al., 2007). Several studies have demonstrated the decline of the photosynthetic process with long-term exposure of plants to an increased CO_2 concentration (Delucia et al., 1985; Thompson et al., 2017; Li et al., 2019). The photosynthetic induction and post illumination CO_2 assimilation responses are affected by the increased CO_2 levels. Due to secondary responses, a surplus carbohydrate aggregation or declined nitrogen (N) content can cause a downregulation of responses of RuBisCo activation (V_{cmax}), stomatal conductance and electron transport (J_{max}) (Thompson et al., 2017). The excess starch that is being created obstructs the CO_2 diffusion and the accumulation of the carbohydrates in the leaves can cause repression of photosynthetic gene expression (Makino & Mae, 1999; Hussein et al., 2021). The photosynthesis suppression caused by the enrichment of CO_2 is often related to the decrease in leaf RuBisCo and leaf N, this is caused by the decrease in N distribution throughout the plant (Smith et al., 2019; Zheng et al. 2019). According to the model by Farquhar et al. (1980), increased CO_2 concentrations affect only V_{cmax} negatively, while increased air temperature showed negative long-term effects on both V_{cmax} and J_{max}. Less directly, the related higher temperatures cause an increase in the plant's transpiration and less water is available for the plant. Degraded soil microbes result in further reduction of photosynthesis in the long-term, as less chlorophyll can be produced (Hussein et al., 2021).

2.3 Leaf structural traits

The Leaf Mass per Area (LMA) is a key indicator for plant growth and the plant's strategies (Grime, 2001; Westoby et al., 2002; Poorter et al., 2009; Asner et al., 2011). The LMA ratio of leaf dry mass to leaf area (g/m^2) is a key feature in understanding plant light uptake and carbon gain, providing information on the process of photosynthesis (Gutschick & Wiegel, 1988; Cheng et al., 2014). The LMA index varies greatly among species and indicates how nutrients are allocated to a certain area of light-intercepting foliage (Poorter et al., 2009). The index Specific Leaf Area (SLA) is the inverse of the Leaf Mass per Area (LMA) and has the unit m^2/kg. The SLA index describes the allocation of leaf biomass per unit of leaf area, which is an important link between vegetation water and carbon cycles (Pierce et al., 1994). Also, the SLA index reflects the expected return on previously captured resources, and consequently a relatively high SLA index is linked to a productive leaf (Poorter & Van der Werf, 1988). On the other hand, leaves with a low SLA index perform better in resource-constrained environments where the retention of acquired resources is a higher priority (Wilson et al., 1999). Plants

modify their SLA index in response to both N-limitation and shade, two elements that interact significantly (Meziane & Shipley, 1999). Shading enhances SLA, which improves shaded plants' ability to capture irradiance (Björkman, 1981; Meziane & Shipley, 1999).

2.4 Leaf nutrient content

The two most significant elements that restrict plant growth, maintenance, and reproduction in terrestrial ecosystems are the macronutrients phosphorus (P) and nitrogen (N) (Reich and Oleksyn, 2004; Elser et al., 2007). According to LeBauer & Treseder (2008), plant N is closely related to photosynthesis, litter decomposition, and plant productivity. Furthermore, genetic processing, energy storage, and membrane structure all depend vitally on plant P (Elser et al., 2007; Reich et al., 2009). The fertilisation effect of increased CO_2 creates the potential to reduce the nutritional value of crops (Mcgrath & Lobell, 2013; Loladze, 2014; Myers et al., 2014). According to a modelling study by Beach et al. (2019), CO_2 fertilisation can cause a decrease of 13,6% for iron and 14,6% for zinc by 2050. Additionally, Myers et al. (2014) stated that the elevated CO_2 concentrations mainly influence the concentration of the micronutrients zinc (Zn), iron (Fe) and calcium (Ca) in the plant's foliar and edible tissues. Dr. Klaus Kraemer (personal communication, May 13, 2022) stated that these three micronutrients are essential for human nutrition and are prone for the impact of the changing photosynthetic traits. Due to these factors, this study will concentrate on these three micronutrients.

In plants, Zn plays a key role as a structural constituent and regulatory cofactor of a wide range of different enzymes and proteins in many biochemical pathways (Alloway, 2008). For human nutrition, Zn is an essential micronutrient for the metabolism that catalyses more than 100 enzymes, facilitates protein folding, and helps regulate gene expression (Saper & Rash, 2009). A reduction in micronutrients could possibly result in 175 million people becoming Zn deficient, if the current trajectory of 550 ppm (parts per million) by 2050 is maintained (Smith & Myers, 2018). One of the most crucial minerals for the growth and development of plants is iron. It contributes to a variety of essential metabolic processes, such as photosynthesis, respiration, and amino acid biosynthesis, as an enzyme cofactor or part of electron transport chains (Gao & Dubos, 2021). For human nutrition, iron is an essential micronutrient as it is involved in a wide variety of metabolic processes, including oxygen transport, deoxyribonucleic acid (DNA) synthesis and electron transport (Abbaspour et al., 2014). Calcium (Ca) is a critical structural and signalling nutrient for plants, and its lack can lead to poor biotic and abiotic stress tolerance, as well as lower crop quality and production. For humans, a low Ca intake has been associated with a variety of disorders (osteoporosis, hypertension, and colorectal cancer) (Dayod et al., 2010).

2.5 Aim, research questions and hypothesis

Prior research has provided evidence on how the photosynthetic traits can be predicted by the N and P concentration in plants. However, the effect of the photosynthesis traits on N and P and on the micronutrients relevant for human nutrition iron, zinc and calcium remains unclear. The photosynthetic traits V_{cmax} and J_{max} are directly linked to the concentration of atmospheric CO_2, thus understanding the relation between the photosynthetic traits and the plants nutrients can provide insights as to how atmospheric CO_2 impacts the nutrient content of a plant. This research aims to investigate the relationship between the photosynthesis traits J_{max} and V_{cmax} and the concentration of leaf nitrogen, phosphorus, iron, zinc and calcium of four different plant species. Scientifically this is relevant as this will improve comprehension of how the photosynthesis performance of a plant is linked to plant growth and nutrient content. For society this is relevant as this link will have an impact on human nutrition. Understanding plants' coping mechanisms with increasing atmospheric CO_2 concentrations is crucial to be able to feed the global rising populations without nutrient deficiencies.

This aim results in the following research question, the sub questions and the corresponding hypothesis:

Research question: How are the photosynthesis traits (V_{cmax}, J_{max}) and the plant's nutrient content (N, P, Fe, Zn, Ca) related and does this differ per species?

SQ1: Do the values for the photosynthesis traits (V_{cmax} and J_{max}), N and P significantly differ per species group?

    H1: For V_{cmax} and J_{max}, Alfalfa > other species. For N and P, there is no significant variation between species.

SQ2: Do plants show consistent stoichiometry when comparing Zn, Fe and Ca absolute values to their ratio with N and P?

    H2: The species variability for ratios < absolute values.

SQ3: How are the photosynthesis traits V_{cmax}, J_{max} related to N and P?

    H3: V_{cmax} and J_{max} are positively related with N and P.

SQ4: How are N and P related to the micronutrients Zn, Fe and Ca?

    H4: N and P are positively related to Zn, Fe and Ca.

Materials and methods

3.1 Data material LIAISE project

To answer the research question, an elemental analysis combined with photosynthesis performance measurements have been done on plant material derived from the LIAISE project in 2021. The LIAISE (Land Surface Interactions with the Atmosphere over Iberian Semi-arid Environment) project retrieved data from the Ebro basin in north-eastern Spain, which is bound to the north by the Pyrenees and to the south by the Iberian system. This particular area was focused on because of the surface heterogeneity, which has increased as a result of human society's alteration of the hydrological cycle and landscape, mostly through intensive agricultural activity (Boone, 2019). The soil is a confounding variable, meaning that only plant material will be included that has grown under conditions containing sufficient nutrients and water. This research has performed the different measurements on the leaves from the same sample material, which contain the following species: grape, alfalfa, almond and apple (2 groups: irrigated and non-irrigated) (see table 1). The apple species have been grown in the IRTA (Institute of Agrifood Research and Technology) experimental facility, as additional measurements (lysimeters) have been done on this species, these measurements are not relevant for this research. All these species are C_3-photosynthesis plants.

Table 1: Plant material species, the derived location and date, sample count and information on irrigation.

Species	Area	Latitude (N) / Longitude (E)	Date retrieved	Irrigated	Sample size
Alfalfa	La Cendrosa	41°69'33.6"N 0°92'84.1"E	17/7/2021	No	5
Almond	Preixana	41°59'37.3"N 1°07'25.0"E	21/7/2021	No	5
Apple	Mollerussa IRTA experimental facility	41°61'76.4"N 0°87'19.7"E	15/7/2021 and 22/7/2021	Yes	9
Apple	Mollerussa IRTA experimental facility	41°61'76.4"N 0°87'19.7"E	15/7/2021, 22/7/2021 and 23/7/2021	No	10
Grape	Vineyard near Verdù	41°59'37.3"N 1°12'72.2"E	20/7/2021	Yes	5

3.2 Analysis of material

Ecophysiological analysis

To measure the photosynthesis traits of the plant material, the LI-6400XT machine was used in the field in the research area in Spain. This machine measures the leaf gas exchange, and provides data on CO_2 response curves to derive the photosynthetic traits V_{cmax} and J_{max} ($\mu mol/m^2/s$). For this research, the data derived from the response curves has been fitted into the enzyme kinetic photosynthesis model developed by Farquhar et al. (1980). By measuring a plant's reactivity to atmospheric CO_2 concentrations and fitting equations to parts of the A/Ci curve, the photosynthetic traits can be calculated (Von Caemmerer & Farquhar, 1981; Sharkey et al., 2007).

Elemental analysis: Picofox

For the elemental analysis, the material from the LIAISE project had to be labelled, dried, grounded and prepared. The samples were labelled as following: day of retrieving sample - species - number of species (as an example: 17Ap1). For the Picofox duplos have been made, so per sample two data points have been retrieved (example: 17Ap1.1 and 17Ap1.2). The plant material has been dried in the oven for 24 hours at 60 degrees. Afterwards the leaf was prepared for grinding by inserting pieces of the leaf in an Eppendorf cup that could be inserted in the laboratory ball mill. Per cup, two metal balls were added to assure adequate grinding. The machine was set to centrifuge for 1500 rpm / 30 Hz, this way the solid leaf material was ground to a powder. The ground (powder) samples were weighed out (50 mg, 4 decimals, as the exact weight is crucial for the elemental analysis) in a cupping glass. After the preparation of the material, the samples were ready for the elemental analysis. This was done by using the Picofox, a transportable TXRF spectrometer for ultra-trace element analysis. With total reflection X-ray fluorescence, the Picofox can give results on the presence and quantity of micronutrients in the samples (Saaltink et al., 2018). All samples had to be put on Quartz discs that go into a tray (24 disks at one time) in the Picofox machine. Before a droplet of the sample could be put on the disc, the disc had to be prepared by coating the top of the disc (which states: 'Bruker Nano') with 10 µL Silica oil (Serva) by using a pipette. The slide was put on a special hotplate to dry. This way, the sample will be properly dispersed over the disc. The method for preparing the discs, applying the sample, the usage of the Picofox machine and cleaning of the discs can be found in appendix A.

Elemental analysis: carbon and nitrogen

For measuring the mass fraction of C and N in the samples, the elemental analyser 'EA IsoLink IRMS system' has been used. The system includes Thermo Scientific™ Flash IRMS™ Elemental Analyser, the Thermo Scientific™ ConFlo IV™ Universal Interface and a Thermo Scientific™ Isotope Ratio Mass Spectrometer. The prepared samples (labelled, dried, ground, weighed in a similar way to the Picofox preparation) were weighed into a tin container and put into the autosampler. When put into the

machine, the sample falls from the sampler into a combustion tube, where it burns in a flash under the influence of oxygen (flash combustion) at 1020 °C. The combustion gases are moved further through the machine with the help of the carrier gas helium to a column of copper oxide where they are completely converted into CO_2, N_2, NO_x, H_2O, SO_2 and residual O_2. Afterwards, the gases flow through a Cu column, where nitrogen oxides are reduced to elemental nitrogen, and O_2 to CuO. Now the water can be absorbed by the column. The gases flow to a TPD (Temperature Programmed Desorption) column. CO_2, H_2O and SO_2 are absorbed and with the help of programmed heating the gases are released one after the other. The gases flow through a thermal conductivity detector (TCD), which emits an electrical signal proportional to the concentration of nitrogen, carbon, hydrogen and sulphur. Before the elemental CN analyser could be used, a calibration curve had to be made (see appendix B). In most measurement methods, instrument calibration is required. It is a set of prescribed operations that establishes the relationship between the measuring system's output (e.g., an instrument's response) and the calibration standards' recognized values (e.g., the amount of analyte present) (Prichard & Barwick, 2003).

Leaf traits calculation: SLA and LMA index

To calculate the SLA and LMA index, the following method was used. First, dried leaves were weighed to retrieve the weight per leaf for each species. At least five leaves were weighed and the average of these values were used for further calculation. To obtain the area of the leaf material, scans have been made of the leaves. With the program ImageJ, the scans have been analysed using the 'Analyse Particles' tool. Having obtained this data for all species, the SLA index could be obtained by dividing the area (m^2) by the weight (kg). The LMA index was calculated by dividing the weight (g) by the area (m^2). The SLA and LMA index have been used to convert the concentrations from weight-basis (g/kg) to area-basis (g/m^2).

3.3 Statistical Analysis

First, a statistical calculation has been made to locate outliers by using the IQR method (Jeong et al., 2017). Afterwards, to validate this method, scatterplots of the first and second elemental analysis measurement per sample have been made in the dataset to visualise the data and a possible regression line. This has been done by using SPSS with the scatterplot function. Having created a dataset for the samples, containing the results from the elemental analysis (from the Picofox and CN analyser), the CO_2 response curves (Farquhar et al., 1980) and the leaf traits with the SLA and LMA index, the statistical analysis could be performed.

The statistical data analysis has been performed in the statistical software platform IBM SPSS Statistics 24.0. For this analysis, analysis of variance (ANOVA) and analysis of covariance (ANCOVA) tests have been performed, as we want to know how the multiple covariates (V_{cmax}, J_{max}, N, P) influence the concentration and ratio of the nutrients and identify possible differences between species. The test result is considered significant if the P-value $= < 0.05$. The ANOVA tests give information on the variance between means, and in this research will describe the variance between species for a certain variable (photosynthetic trait or nutrient). The ANCOVA test can give information on the regression analysis, which variable can be a possible predictor of another variable. A supplementary Tukey HSD post-hoc test has been performed, to obtain knowledge about which variable differs significantly from another variable. Additionally, a F-test is used to compare the magnitude of the variability in the absolute micronutrient values and in the ratios of the micronutrients with N and P. Per sub question, one or multiple statistical tests will be performed:

SQ1: Do the values for the photosynthesis traits (V_{cmax} and J_{max}), N and P significantly differ per species group?

> **Statistical approach:** performance of multiple ANOVA and ANCOVA tests for the following variables: V_{cmax}, J_{max}, N, P. To visualise the variation between species, boxplots have been made. To improve understanding and visualise the dataset, scatterplots have been made which include a R^2 value for the photosynthetic traits and for the units g/m^2 and g/kg for N and P.

SQ2: Do plants show consistent stoichiometry when comparing Zn, Fe and Ca absolute values to their ratio with N and P?

> **Statistical approach:** performance of multiple ANOVA tests for the following variables: Zn, Zn:N, Zn:P Fe, Fe:N, Fe:P, Ca, Ca:N, Ca:P. The comparison of the P-value results of the ANOVA tests give insights into the rate of variation between species for absolute values and ratios. Also, F-tests have been performed to analyse the difference in magnitude of the variability, which provide a F-value and P-value.

SQ3: How are the photosynthesis traits V_{cmax}, J_{max} related to N and P?

> **Statistical approach:** performance of multiple ANCOVA tests for V_{cmax} and J_{max} as covariates for N and for P. To visualise the relations, scatterplots have been made that also bring insight into a positive or negative relation and provide a R^2 value.

SQ4: How are N and P related to the micronutrients Zn, Fe and Ca?

> **Statistical approach:** performance of multiple ANCOVA tests for P and N as covariates for Zn, Zn:N, Fe, Fe:N, Ca and Ca:N. To visualise the relations, scatterplots have been made that also bring insight into a positive / negative relation and provide a R^2 value.

Results

4.1 Variation for photosynthetic traits, nitrogen and phosphorus

First, the two photosynthetic traits V_{cmax} and J_{max} have been plotted in a scatterplot (figure 1) to obtain the R^2 value per species group. A difference between the species can be observed, for alfalfa (R^2 of 0.928) and almond (R^2 of 0.970), the high R^2 explains a strong correlation. For irrigated apples (R^2 of 0.611) and grapes (R^2 of 0.595) the medium R^2 explains a moderate correlation, and for non-irrigated apples (R^2 of 0.269) the low R^2 explains a weak correlation. The ANCOVA test result is significant for J_{max} as covariate for V_{cmax} (P-value = $3.25*10^{-11} < 0.05$), and reversely, the effect of V_{cmax} on J_{max} is significant with a P-value of $1.65*10^{-9}$.
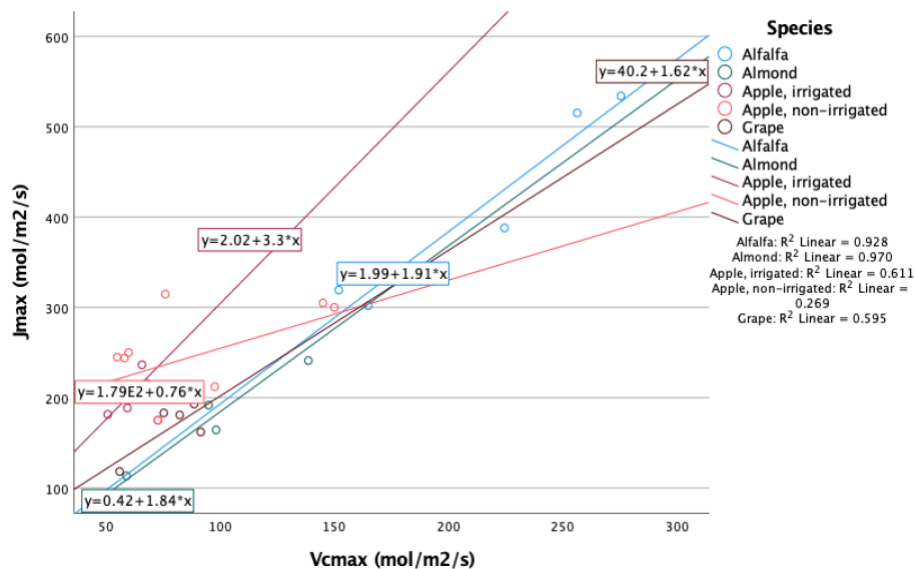

Figure 1: Scatterplot of the photosynthetic traits V_{cmax} and J_{max} (μmol/m^2/s), subdivided per species. The regression equation is attached to each regression line and R^2 values are added to the legend. The P-value for J_{max} as covariate for V_{cmax} is significant (P=$3.25*10^{-11} < 0.05$). Also, the P-value for V_{cmax} as covariate for J_{max} is significant (P=$1.65*10^{-9} < 0.05$). A positive correlation can be concluded between V_{cmax} and J_{max}.

The variation between species of the photosynthesis traits and nitrogen and phosphorus has been tested with an 'Analysis of Variance' (ANOVA) test. Both the photosynthesis traits vary enough between species to obtain a significant result. The P-value is $3.36*10^{-6}$ for V_{cmax} and $1.58*10^{-4}$ for J_{max}. Figure 2 visualises the significantly higher concentration for V_{cmax} and J_{max} for alfalfa compared to other species, which is also tested in the Tukey HSD post-hoc test.
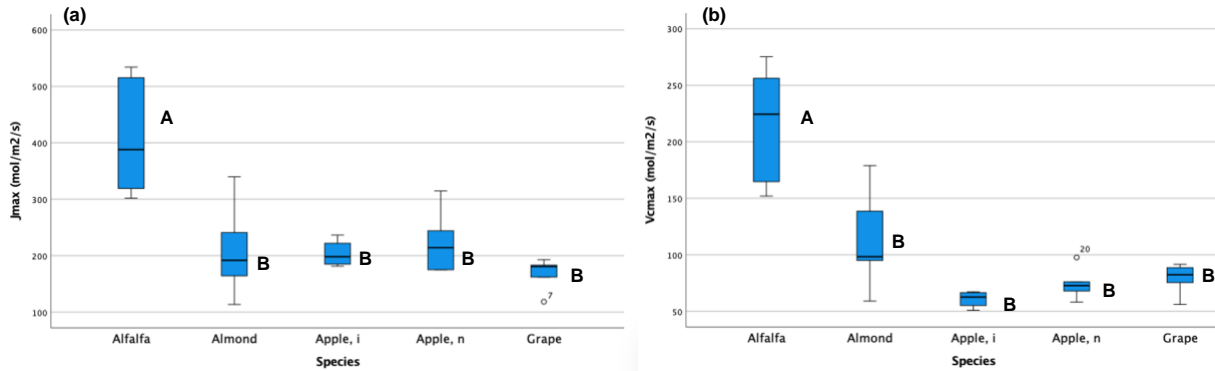
Figure 2: The boxplots visualise the means of the photosynthetic traits J_{max} (a) and V_{cmax} (b) in µmol/m^2/s for species. The P-value is $3.36*10^{-6}$ for V_{cmax} and $1.58*10^{-4}$ for J_{max}, both significant as $P < 0.05$. The box shows the values between the 25th and the 75th quartile and the black line in the box is describing the median. The letters describe the different groups, explaining group A varies significantly in mean from group B.

When plotting N and P concentrations in the units g/m^2 and g/kg in a scatterplot, a perfect fit can be observed (figure 3). The R^2 value is 1 for both N and P for all species, meaning that the response variable can be perfectly explained without error by the predictor variable. In this case, the N/P (g/kg) can be perfectly explained by N/P (g/m^2). Because of this reason, and because the unit for the photosynthesis traits is in area basis (µmol/m^2/s) the analysis of (co)variance tests have been performed with the g/m^2 unit.
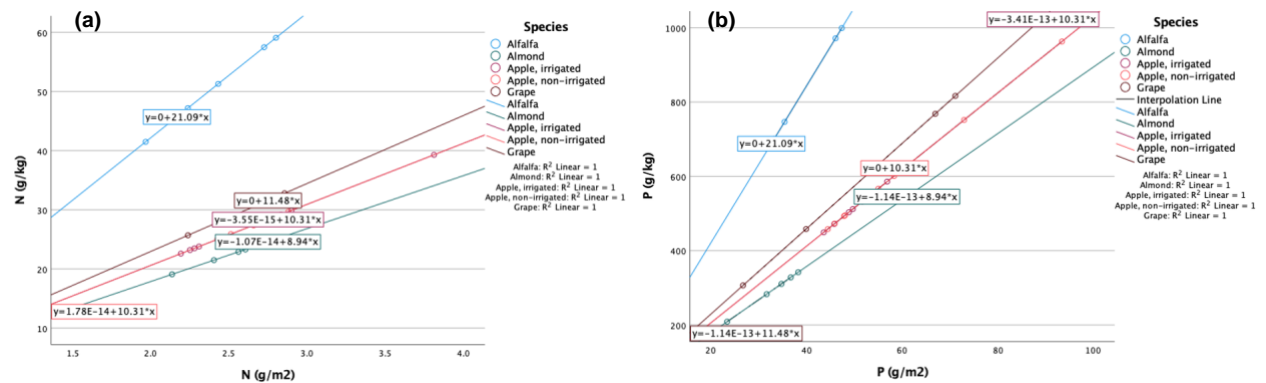

Figure 3: Scatterplot of nitrogen in two units (g/kg and g/m^2) (a) and phosphorus in two units (g/kg and g/m^2) (b), subdivided per species. The regression equation is attached to each regression line and R^2 values are added to the legend. The values for R^2 are all 1, describing X can perfectly be explained by Y.

To analyse the variance between species for N and P concentration, an ANOVA test has been performed. The P-value for N (g/m^2) is 0.551 which is > 0.05 (result not significant). Figure 4 visualises the low variation in N concentration between species. The P-value for P is $4.79*10^{-3}$, which describes a significant variation between species for P g/m^2 concentration. This result is significant because of the difference in concentration between almond and non-irrigated apples and because of the difference between almonds and grapes. The ANOVA test for N:P ratio gives a P-value of $0.021 < 0.05$. The post-hoc test is not significant, explaining not a significant variation between species for the N:P ratio.
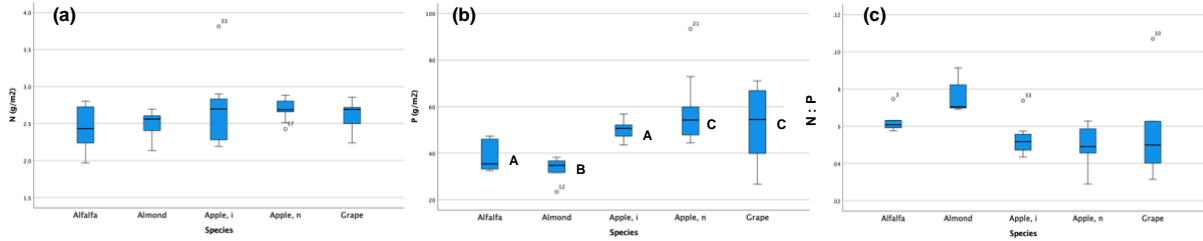
Figure 4: Means of N (a) and P (b) concentration for g/m^2 and ratio N:P (c) in boxplot for species. The P-value for N (g/m^2) is 0.551 > 0.05 (not significant), because of the low variation between species. The P-value for P (g/m^2) is 4.79*10^{-3} < 0.05 (significant) and the post-hoc Tukey test is significant. The P-value for the N:P ratio is 0.021<0.05 (significant), the post-hoc Tukey HSD test is not significant. The box shows the values between the 25th and the 75th quartile. The black line in the box is describing the median. The letters describe the different groups, explaining group A varies significantly in mean from group B, and C differs significantly from group A and from group B.

4.2 Micronutrients stoichiometry

To answer the second sub question, and to determine if the variability is lower with the ratios with N and P compared to the variability with the absolute values, a F-test was performed which uses the standard deviation and the variance. For iron, the standard deviation is 6.721 and the variance 45,171. For the Fe:N ratio, the standard deviation is 2.497 and the variance is 6.235. The F-test gives the following results for the variation between Fe and Fe:N: F-value is 2.692 and P-value is 0.006. When performing the test on the Fe:P ratio, the standard deviation is 0.171 and the variance is 0.029. The F-test, which compares the standard deviation of Fe and Fe:P gives a F-value of 39.304 and a P-value of <0.001. These results describe a decrease in variability for the ratio with nitrogen and a further decrease for the ratio with phosphorus compared to the variability of the absolute Fe value.

Additionally, an ANOVA test has been performed to research the variance in means between the species for absolute micronutrient values and ratios with nitrogen and with phosphorus, and boxplots are included for the visualisation of the results. A low P-value describes a high variation. For iron absolute value (g/m^2), the P-value is 5.16*10^{-5}. The post-hoc Tukey HSD test is not significant (P-value 0.192 > 0.05). For the Fe:N ratio, the P-value is 2.22*10^{-4}, explaining a significant variation between species. Also here, the post-hoc Tukey HSD test is not significant (P-value is 0.362 > 0.05), so the variation cannot be linked to a certain species. For Fe the variation is lower for the ratio with N compared to the absolute value (P-value of 5.122*10^{-5} < 2.254*10^{-4}). The ANOVA test for Fe:P gives a significant P-value of 0.007. However, the post-hoc Tukey HSD test is not significant (P-value is 0.073 > 0.05), so the variation cannot be linked to a certain species. To conclude, all ANOVA tests are significant, and the variation between species is highest for the iron absolute values. The lowest variation between species is found at Fe:P. Note the step sizes of the boxplots (figure 5), the step size decreases going from absolute value to ratio with N and a further decrease in step size for the ratio with P.
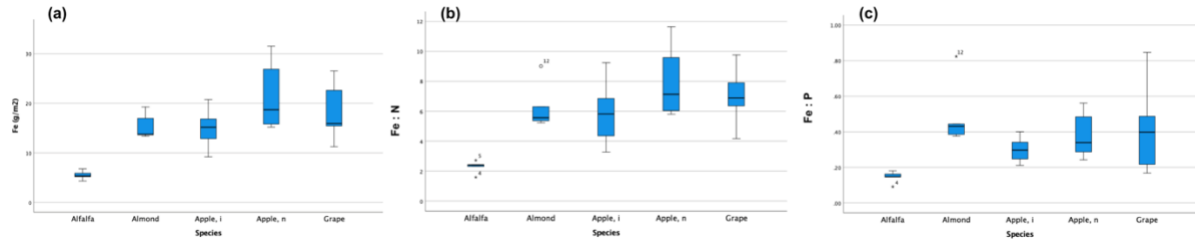
Figure 5: Boxplot of Fe absolute concentration for g/m^2 (a), Fe:N ratio (b) and Fe:P ratio (c) in boxplots for species. The P-value for Fe (g/m^2) is $5.16*10^{-5} < 0.05$ (significant). For Fe:N, the P-value is $2.22*10^{-4} < 0.05$ (significant) and for Fe:P the P-value is $0.007 < 0.05$ (significant). The post-hoc Tukey HSD is not significant for Fe (g/m^2), Fe:N and Fe:P. The box shows the values between the 25th and the 75th quartile. The black line in the box is describing the median. Note the step size of the Y-axis decreasing with the ratio with N and further decreasing with P.

Similar tests have been performed for Zn and Ca concentrations. First, the standard deviation and variance for Zn and for Zn ratios with N and P have been obtained. The standard deviation for the absolute Zn value is 0.486, and the variance is 0.237. The standard deviation for Zn:N is 0.197 and the variance is $3.899*10^{-2}$. The F-test result for Zn and Zn:N is 2.467 and the P-value is 0.011. For the Zn:P ratio, the standard deviation is 0.012 and the variance is $1.535*10^{-4}$. The F-test result between Zn and Zn:P gives a F-value of 39.256 and a P-value of <0.001. The standard deviation and the variance decrease for the ratios compared to the absolute Zn value, and are lowest for the Zn:P ratio.

When performing the ANOVA test to analyse the variance between the means between species, the test for Zn gives a P-value of $3.551*10^{-2}$. The post-hoc Tukey HSD test is not significant (P=0.501>0.05). The ANOVA test for the Zn:N ratio gives a P-value of $2.748*10^{-2}$. The post-hoc Tukey HSD test is again not significant, thus this significant variation cannot be linked to a certain species. The P-value for the Zn:P concentration is $4.366*10^{-6}$. Also here, the post-hoc Tukey HSD test is not significant. For Zn, the P-value for the absolute concentration is higher compared to the P-value of the ratio with N, and also higher compared to the ratio with P. To conclude, the variation for the absolute Zn concentration is slightly lower compared to the ratios with N and P. Similar to the comparison for Fe, note the decrease in step sizes (figure 6) for the ratios compared to the absolute values.
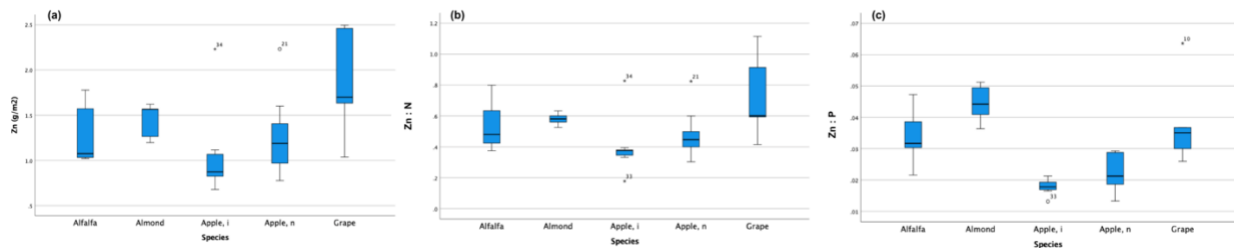

Figure 6: Boxplot of Zn absolute concentration for g/m^2 (a), Zn:N ratio (b) and Zn:P (c) in boxplots for species. The P-value for Zn (g/m^2) is $3.551*10^{-2}$ <0.05 (significant). For the Zn:N ratio, the P-value is $2.748*10^{-2}$ <0.05 (significant). The P-value for the Zn:P ratio is $4.366*10^{-6}$ <0.05 (significant). All post-hoc Tukey HSD tests are not significant. The box shows the values between the 25th and the 75th quartile. The black line in the box is describing the median. Note the step size of the Y-axis decreasing with the ratio with N and further decreasing with P.

The standard deviation for Ca is 0.617 and the variance is 0.381. The standard deviation for the ratio Ca:N is 0.259 and gives a variance of 0.066. The F-test for Ca and Ca:N gives a F-value of 2.387 and a P-value of 0.007. For Ca:P, the standard deviation is 0.022 and the variance $5.12*10^{-4}$. The F-test result for comparing the standard deviation between Ca and Ca:P gives an F-value of 27.221 and a P-value of <0.001. The variability is lower for the ratios with N and P, and lowest for Ca:P.

To obtain results on the variance between the species, the ANOVA test has been performed and gives a P-value is $6.789*10^{-10}$ for the absolute Ca concentration, the post-hoc Tukey HSD test is not significant, thus this significant variation cannot be linked to a particular species. The Ca:N ANOVA test gives a P-value of $2.621*10^{-7}$, and the post-hoc Tukey HSD test is also not significant. The P-value for the Ca:P ratio is $2.213*10^{-6}$. The post-hoc Tukey HSD test is not significant. For Ca the variation between species is lower with the Ca:N ratio compared to the Ca absolute value, and the Ca:P ratio contains the lowest variation.
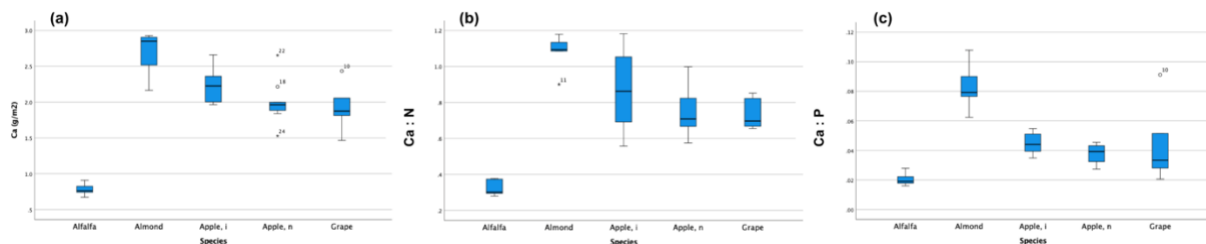

Figure 7: Boxplot of Ca absolute concentration for g/m^2 (a), Ca:N ratio (b) and Ca:P ratio (c), in boxplots for species. The P-value for Ca (g/m^2) is $6.789*10^{-10} < 0.05$ (significant). The P-value for Ca:N is $2.621*10^{-6} < 0.05$ (significant) and for Ca:P is $2.213*10^{-6} < 0.05$ (significant). All post-hoc Tukey HSD tests are not significant. The box shows the values between the 25th and the 75th quartile. The black line in the box is describing the median. Note the step size of the Y-axis decreasing with the ratio with N and further decreasing with P.

4.3 Photosynthetic traits relation to nitrogen and phosphorus

To understand if the values of N and P can be predicted by the photosynthetic traits values, analysis of covariance (ANCOVA) tests have been performed. For N (g/m^2), the test gives no significant result, explaining no significant effect of V_{cmax} and J_{max} on concentration of N (g/m^2). Also with this test, no significant N variation between species can be found as the result of this test is not significant, explaining a low variation between species for N (g/m^2).

Secondly, the ANCOVA test has been performed for V_{cmax}, J_{max} and P. A significant effect was found of the covariates V_{cmax}, J_{max} on P (g/m^2). So, the concentration of P (g/m^2) in the leaves can be predicted out of V_{cmax}, J_{max}. The P-value for V_{cmax} as covariate for P is $3.973*10^{-3} < 0.05$. The P-value for V_{cmax} as covariate for P is $1.256*10^{-2}$. Also, after correcting for V_{cmax}, J_{max}, there is a significant difference between P (g/m^2) for species (P-value $=8.792*10^{-4}$). The result is significant because of the difference in concentration between almond and non-irrigated apple and because of the difference between almond

and grape (see figure 4b). To visualise the significant result of the ANCOVA test for V_{cmax}, J_{max} and P, two scatterplots have been made. The first scatterplot (figure 8a) with J_{max} (μmol/m^2/s) and P (g/m^2) shows a positive correlation for almond (R^2 of 0.145), and a negative correlation for the other species (non-irrigated apples $R^2 = 0.645$, irrigated apples $R^2 = 0.653$, alfalfa $R^2 = 0.283$, grape $R^2 = 0.454$). The second scatterplot (figure 8b) shows the results for V_{cmax} and the P (g/m^2). Here, non-irrigated apple, grape and alfalfa give a low R^2 value (respectively $R^2 = 4.214*10^{-6}$, $R^2 = 0.065$, $R^2 = 0.090$). Both irrigated apple ($R^2 = 0.886$) and almond ($R^2 = 0.191$) show a negative correlation between V_{cmax} and P (g/m^2).
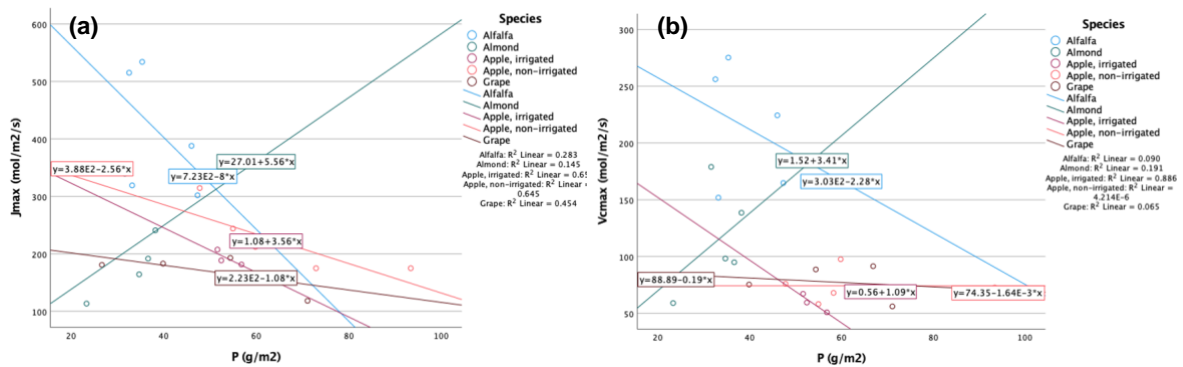

Figure 8: Scatterplot of P (g/m^2) and the photosynthetic traits J_{max} (a) and V_{cmax} (b), subdivided per species. The regression equation is attached to each regression line and R^2 values are added to the legend. The P-value for the ANCOVA test for J_{max} as covariate for P is $3.973*10^{-3}$, and for V_{cmax} as covariate for P, the P-value is $1.256*10^{-2}$. Both J_{max} and V_{cmax} are significant covariates. The species covariate for P (g/m^2) concentration is significant with a P-value of $8.792*10^{-4}$.

4.4 Relationship between nitrogen, phosphorus and micronutrients

To understand the relationship between N, P and the micronutrients, several ANCOVA tests have been performed. The tests have been done for the g/m^2 unit and for the ratio with N and P. For Fe, it was tested if the covariates N (g/m^2) and P (g/m^2) impact the concentration of the dependent variable Fe (g/m^2). Results of the ANCOVA test are not significant. So, Fe (g/m^2) cannot be predicted by the covariates because there is no significant impact. Also, when performing the test with the Fe:N and Fe:P ratio, the ANCOVA test results are not significant, describing that the Fe:N and Fe:P ratios cannot be predicted by the covariates N and P.

A similar procedure has been used for Zn. This ANCOVA test was significant (P-value = 0.003 < 0.05), and when looking at the significance of the covariates, a significant result was seen with P (g/m^2) and Zn (g/m^2) (P-value = $3.912*10^{-2}$ < 0.05). For N (g/m^2) and Zn (g/m^2), there was no significant result. The positive correlation between Zn and P can be seen in figure 9. The R^2 is highest for grapes (0.571). R^2 for almond is 0.477, for non-irrigated apple $R^2 = 0.441$, for irrigated apple $R^2 = 0.310$ and for alfalfa

$R^2 = 0.039$. The covariate species is not significant (P-value = 0.072 > 0.05), meaning that the sort of species does not impact the Zn - P relationship.

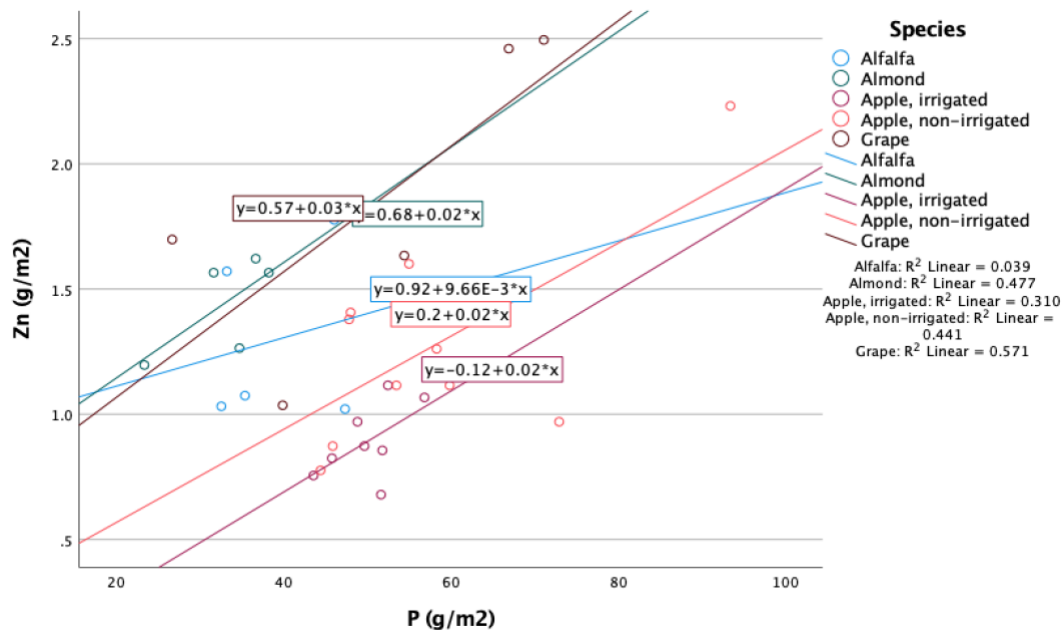**Figure 9:** Regression line for P (g/m^2) and Zn (g/m^2). The regression equation is attached to each regression line and R^2 values are added to the legend. The P-value for P (g/m^2) as covariate for Zn (g/m^2) is significant (P-value = 3.912*10^{-2}). The covariate species is not significant (P-value = 0.072 > 0.05).

When performing the analysis of the covariance test for P and N as covariates for Zn:N ratio, a significant impact was found (P-value = 0.009 < 0.05). When performing this test, the covariates species also gave a significant result (P-value = 0.005 < 0.05). This can be explained by the significant difference between almond and irrigated apple (P-value = 0.019 < 0.05), and the significant difference between grape and irrigated apple (P-value = 0.008 < 0.05). This can be explained by the lower value of irrigated apples compared to almonds and grapes. Also, a significant difference between almond and non-irrigated apple (P-value = 0.063 < 0.05) was found. This can be explained by the lower value of Zn:N or non-irrigated apple compared to almond.

Lastly, an ANCOVA test has been performed for Ca with covariates N, P and species. The results of the test are not significant for the covariates N and P for the absolute value of Ca, meaning N and P cannot predict the concentration of Ca (g/m^2). However, a significant difference can be seen for Ca concentration between species, due to the relatively low concentration of Ca in alfalfa compared to the other species. For the ratio Ca:N, no significant effect is found from the covariates N and P. However, a significant difference can be found between species for the Ca:N ratio, due to the low concentration of Ca:N in the alfalfa species, compared to all non-irrigated apples, grapes and almonds. The difference in Ca:N concentration between alfalfa and irrigated apples is not significant.

Discussion

5.1 Interpretation of results

The aim of this research was to improve understanding of how nutrient content in plants can be predicted by the photosynthetic traits. The following research question was set up: How are the photosynthesis traits (V_{cmax}, J_{max}) and the plant's nutrient content (N, P, Fe, Zn, Ca) related and does this differ per species? To answer the research question and sub questions, statistical analysis of (co)variance tests have been performed on the data that has been obtained by elemental analysis and photosynthetic performance measurements.

To answer the first sub question, the relationship between the photosynthetic traits have been tested. Prior research has provided evidence on the strong relationship between J_{max} and V_{cmax} (Wullschleger, 1993; Beerling & Quick 1995). This research's findings are similar compared to previous research. The first SQ and corresponding hypothesis were formulated as follows:

SQ1: Do the values for the photosynthesis traits (V_{cmax} and J_{max}), N and P significantly differ per species group?

> **H1:** For V_{cmax} and $J_{max,}$ Alfalfa > other species. For N and P, there is no significant variation between species.

For both V_{cmax} and J_{max}, alfalfa contains a significantly higher value compared to the other species. This was hypothesised as alfalfa's leaf size is smaller compared to the other species. According to prior research, smaller leaf sizes correspond with higher photosynthetic activity, resulting in higher V_{cmax} and J_{max} values (Bronstein et al., 2007). For N, no significant variance was found which corresponds with the hypothesis set up for N, which states that there is no significant variation between species for N. However, the results are not in line with the hypothesis for P, as a significant variation was found between species. The P concentration for almond was significantly lower compared to the P concentration of grape and non-irrigated apple. To conclude, an unexpected variance exists between species for P concentration. The greater variance between species for P concentration compared to N concentration can be explained by previous research done by Kang et al. (2011). This research stated the variation between species is lower for N than for P.

The second sub question compared absolute micronutrient concentrations to the ratios with N. The hypothesis expected the variation between species to be lower for the ratios than for the absolute values. The difference between absolute values and ratios can explain if the stoichiometry is consistent or not, which indicates if nutrients are related to each other or not.

SQ2: Do plants show consistent stoichiometry when comparing Zn, Fe and Ca absolute values to their ratio with N and P?

H2: The species variability for ratios < absolute values.

The results of the comparison of variability with the F-test between ratios and absolute values for Fe, Zn and Ca indicate a decrease in variability for the ratio with N compared to the absolute micronutrient value. The variability for the ratio with P is lowest. This is consistent for all Fe, Zn and Ca and this result agrees with the hypothesis for the second sub question.

The results of the ANOVA test indicate a lower variability for the ratios with N and P for Fe and for Ca, compared to the variability between species for the absolute values of Fe. For the ratio with N, this can be explained because N contains no significant variability between the species, decreasing the variability between species if a ratio is made with N. A low and significant P-value explains a high variability. The post-hoc Tukey HSD test of the Fe:N ratio is not significant, so the variance cannot be explained by a certain species, explaining the lower variance for Fe:N compared to absolute Fe. The P-value for the ratio Fe:P is lower compared to the Fe:N ratio, and this lower P value explains a lower variability. A lower variability for the ratio compared to the absolute value assumes that the micronutrients are linked to the macronutrient, as the stoichiometry is not constant for the absolute values and for the ratios (Fe:P and Fe:N).

For Ca, both the absolute and the Ca:N, Ca:P post-hoc Tukey HSD test are not significant, so the variance here is also not explained by a certain species. The P-values for the ratio:N and for ratio:P for Ca are higher compared to the absolute value, which is in line with the hypothesis. Similar to the case for Fe, the stoichiometry for Ca is not constant for the absolute Ca concentration compared to the ratio with N and with P, this assumes a linkage between the micronutrient Ca and N and P. So, we can expect that Ca will react in a similar way to how N and P will react to changing CO_2 concentrations.

For the micronutrient Zn, the ANOVA results are contradicting with the hypothesis as the P-value of the ratio:N and the ratio:P is lower compared to the P-value of the absolute Zn concentration, however, the difference in P-value is only $8.031*10^{-3}$. For the absolute Zn concentration, the post-hoc Tukey HSD test is significant because of the significantly lower Zn concentration in irrigated apples compared to grapes. The Zn:N and the Zn:P post-hoc test is not significant. As the post-hoc test for the absolute Zn concentration is significant, the variation seems larger compared to the ratios. Because of this minor difference for only the zinc species and because of the result of the F-test, we do accept the hypothesis.

To derive results on the relationship between the photosynthesis traits V_{cmax}, J_{max} and N and P, covariance analysis tests have been performed. The following sub question and hypothesis had been set up:

SQ3: How are the photosynthesis traits V_{cmax}, J_{max} related to N and P?

    H3: V_{cmax} and J_{max} are positively related with N and P.

Prior research has provided evidence on the relation between the photosynthesis traits V_{cmax}, J_{max} and N, because of the large amount of N is invested in the RuBisCo protein (Marschner, 1995; Taiz & Zeiger 2010; Prashar et al., 2013; Walker et al., 2014; Wang et al., 2018). Furthermore, the Farquhar et al. model (1980) showed negative effects of increased CO_2 only affecting V_{cmax}. However, this research was interested in V_{cmax} and J_{max} being predictors for the N/P concentration.

The statistical tests gave no significant result for V_{cmax}, J_{max} as covariance for the N concentration. Thus, this result for N is not in line with the hypothesis. However, the research review by Walker et al. (2014) indicated a positive correlation between N and V_{cmax}, J_{max}. The outcome of the ANCOVA test, which is not in line with previous evidence, could be explained by the low sample size, so for further research it would be interesting to perform a similar method with a larger sample size.

The significant result of the ANCOVA test performed for V_{cmax}, J_{max} and P is in line with the hypothesis as there is a significant relation. Both V_{cmax} and J_{max} are predictors for the P concentration in the leaf of the samples. For V_{cmax} and P, the R^2 values are describing a negative correlation for irrigated apple and almond. For the other species, the R^2 is too low to describe as a clear correlation. For J_{max} and P, all species besides almond show a relatively strong negative correlation. The R^2 for almond is positive and relatively low. As the R^2 values per species are contradicting each other in being positive or negative, these results are not strong enough to be able to state if the correlation is positive or negative. As a prior research review by Walker et al. (2014) of 24 papers and 135 species and location combinations (distributed globally) evidenced the positive correlation between V_{cmax} and J_{max} and P, the results will be interpreted as a positive correlation.

The fourth hypothesis has been tested by performing analysis of covariance tests on N, P and the micronutrients Zn, Fe and Ca. The related sub question was set up as follows:

SQ4: How are N and P related to the micronutrients Zn, Fe and Ca?

    H4: N and P are positively related to Zn, Fe and Ca.

The results of the ANCOVA statistical tests were not significant for P, N and Fe and for P, N and Fe:N. Also, the statistical tests were not significant for P, N and Ca and for P, N and Ca:N. For further research it would be interesting to do similar tests for a larger sample size so see if a possible correlation exists.

The significant results for the statistical covariance analysis for P and Zn and for P and Zn:N can be interpreted as P being a predictor of Zn concentration, and as the correlation is positive, an increase in P results in an increase in Zn. The covariate species was not significant, describing no clear impact of species on the P - Zn relationship. The positive correlation between P and Zn was highest for grape and lowest for alfalfa. This is in line with prior research done by Xie et al. (2019), who provided the evidence for the positive relationship between concentration of micronutrients and leaf size. The increase in P concentration is related to increase in leaf size, resulting in increase in micronutrient concentration.

The aim of this research is to understand the linkages between micro- and macronutrients in a plant's leaf, to be able to make predictions of nutrient content with increasing atmospheric CO_2 concentration. This research contributed to the knowledge gap as the results assume a significant positive correlation between the photosynthetic traits and phosphorus, and a significant positive correlation between phosphorus and zinc. As prior research by Zheng et al. (2019) has proved, CO_2 increase leads to a down-regulation of the photosynthetic traits V_{cmax} and J_{max}. The abundant CO_2 availability for the plant leads to a decrease in stomatal conductance, due to the declines in stomatal density and stomatal area. The down-regulation of the photosynthetic traits is associated with a decrease in phosphorus concentration, and it can be assumed that all micronutrients related to P concentration will experience this down-regulation as well. This research has evidenced significant impact on the zinc concentration, however, it is expected that other micronutrients will perform in a similar way.

5.2 Limitations

Some potential limitations exist in this research, which are important to acknowledge for further research on this topic. First of all, the sample size was relatively low and was limited to four species. Especially for the analysis of covariance tests, a larger sample size would be convenient. For apple, the differences in results for apple irrigated and apple non-irrigated were presumed as large enough to be taken up as two separate groups. This difference in performance can be explained by the plant's habituation to the humidity of the soil due to different irrigation patterns. Secondly, this research focused exclusively on plant leaf material, as this way data on photosynthetic performance and elemental analysis could be derived from the same material. In order to conduct this study, it was necessary to establish assumptions about the homogeneous distribution of nutrients throughout the plant and the representativeness of the leaf's nutritional content. Lastly, when performing the SLA and LMA index calculations, it was needed to scan hydrated leaves. However, the scans were only done on

hydrated leaves for the alfalfa species due to a miscommunication. The other species were in a dehydrated state. To perform this analysis, the dehydrated material had to be rehydrated for 48 hours and scanned afterwards, to assure leaf size of hydrated leaves as this is frequently larger compared to leaf size of dehydrated leaves. It is unlikely that this has led to significant inaccuracies, however, it would be expected that direct scans before dehydration are possibly more accurate compared to scans from a rehydration process.

5.3 Recommendations

In light of the findings of this study, it is recommended that future research performs this method with a larger sample size, and possibly as well with samples from varying terrestrial biomes. Also, as mentioned before as a limitation, it appears worthwhile to perform similar research on the fruits and grains of a plant as these results are more relevant when investigating the impact of changing nutrient content on human health. Further research could perform this analysis on fruit and/or grain material, and potentially compare the results with the leaf material to assess possible differences which could provide information on the homogeneity of nutrient distribution in a species. Furthermore, this research had to make assumptions on the effect of atmospheric CO_2 concentration on the plant's nutrient composition, as only material was available from plants that had grown under similar atmospheric CO_2 concentration circumstances. For future research it would be interesting to grow plants under varying ppm CO_2 concentrations and derive the V_{cmax}, J_{max} and elemental composition of the plant. These additions could improve the validity and credibility of the research.

Conclusion

The major findings of this research contributed to answering the research question: How are the photosynthesis traits (Vcmax, Jmax) and the plant's nutrient content (N, P, Fe, Zn, Ca) related and does this differ per species? The first finding indicates that the photosynthetic traits V_{cmax} and J_{max} predict the concentration of phosphorus. The correlation differs per species and is strongest at V_{cmax} for irrigated and non-irrigated apples and grapes. For J_{max}, the correlation with phosphorus is strongest for irrigated apples. The correlation is interpreted as a positive correlation based on evidence of several prior studies. Secondly, the covariance analysis on phosphorus and zinc is significant, stating a positive correlation between phosphorus and zinc. The covariance analyses for the micronutrients iron and calcium were not significant, however, the analysis of variance and the F-test between species for absolute and ratio values provide insights in the consistency of the stoichiometry. The decreased variation with the ratio with nitrogen and with phosphorus presume a linkage between the micronutrient and the macronutrient. For iron and for calcium the variation for the ratio with nitrogen and with phosphorus was lower compared to the variation between species at the absolute value. These results assume a connection between the micronutrients iron and calcium with N and P.

To conclude, the findings of this research conclude that an increase in atmospheric CO_2 concentration results in the downregulation of the photosynthetic traits V_{cmax} and J_{max}, because of the decrease in stomatal conductance (Lui et al., 2014) and reduced allocation of nitrogen which is essential for the RuBisCo enzyme (Walker et al., 2014). The downregulation of the photosynthetic traits leads to a decrease in leaf P and presumably also in leaf N, even though the results of this statistical analysis for nitrogen were not significant. As previous research provided the evidence of the positive correlation between the photosynthetic traits and N and P, this research builds on this evidence and therefore suggests that the micronutrients Fe, Zn and Ca react to the downregulation of J_{max} and V_{cmax} in a similar way as N and P react.

This research contributed to existing literature by increasing understanding on possible connections between the photosynthetic traits and the impact on the essential micronutrients for human nutrition. Future research should focus on the possible theory that downregulation of the photosynthetic traits results in a decrease in micronutrient content of plants. Ultimately, the impact of atmospheric CO_2 increase on nutrient deficiencies can be fully understood so that preventative measures can be taken to reduce the global threat on nutrient availability.

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Appendix

A: Elemental analysis utilisation

Prepare the sample

To ensure a homogeneous solution, the following steps need to be taken to prepare the sample of 50 µg:

1. Add 4.0 mL Triton 1% solution
2. Add 10.0 µL Selenium (Se) as standard
3. Add small magnet to be used for shaking the solution
4. Put the solution on the magnet

Picofox machine usage

1. First a blank measurement had to be done, cleaned and coated discs were put in a tray that is used for the Picofox machine.
2. Measure the slides for 100 seconds, assigning a standard element, a standard concentration or mass and the sample mass.
3. The peaks were checked, as it is a blanc run, only Silicon, Argon and Molybdenum should have peaks.
4. Now, the samples could be added to the slides by pipetting 10 µL of the sample in the middle of a prepared, cleaned and pre-coated Picofox slide.
5. We created duplo's, so a 10.0 µL droplet of each sample was added to two slides.
6. The samples were dried on a special hotplate (level 1) for 2 minutes.
7. After the samples were all dry, the slides were put in the tray which can hold 24 slides, and this tray could be inserted in the Picofox machine.
8. Create a job on the Picofox computer, put the measure time on 500 seconds and fill in the exact weighted sample amount. Standard amount = 10.0 µg. Units = mg/kg.
9. Check if the high voltage (lamp) is on and start the job (measurement).

Cleaning the discs

1. First, the disks were cleaned with tissue paper with ethanol
2. Afterwards, the disks were placed in the cleaning holder that has been put in a glass beaker with 5% decon solution at 60°C for 1.5 hours.
3. The beaker with the holder was put into an ultrasonic bath for 10 minutes and rinsed with water afterwards.
4. The holder was put into a 10% HNO_3 solution for 1.5 hours.
5. The holder was rinsed thoroughly with demi water
6. After this process, the discs holder was placed in a hot oven (warm up at 80°C) turned off (to exclude air flow) for 30 minutes
7. The cleaned holder with disks was put away in the desiccator to cool down, and can now be used again.

B: Calibration curve for carbon nitrogen analysis

Table 2: calibration curve for the CN analysis

# in machine	Compound	Weight
1	Nicotinamide	0.5 - 1.0 mg
2	Blank	-
3	Acetanilide	0.2 - 2.0 mg
4	Atropine	0.2 - 2.0 mg
5	IVA (Plant Material)	1.0 - 2.0 mg
6-15	Samples	1.0 - 2.0 mg
16	Blank	-
17	Acetanilide	0.2 - 2.0 mg
18	Atropine	0.2 - 2.0 mg
19	IVA (Plant Material)	1.0 - 2.0 mg
20-30	Samples	1.0 - 2.0 mg
31	Blank	-
32	Acetanilide	0.2 - 2.0 mg
33	Atropine	0.2 - 2.0 mg
34	IVA (Plant Material)	1.0 - 2.0 mg

C: Leaf structural traits

The table below shows the SLA and LMA index used to convert the units of the concentration from g/kg to g/m^2.

Table 3: SLA and LMA index

Species	Average weight (kg)	Average area (m2)	**SLA** (m^2/kg)	**LMA** (g/m^2)
Apple	0.0013	0.0138	10.307	97.022
Alfalfa	0.0003	0.0014	21.094	47.406
Almond	0.0007	0.0059	8.941	111.846
Grape	0.0007	0.0081	11.483	87.085