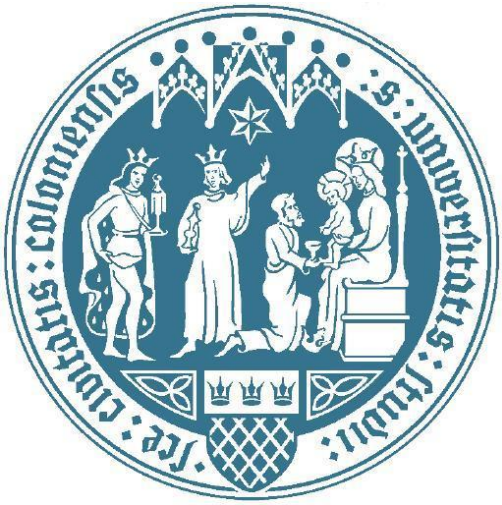

Impact of evolution of C_4 photosynthesis on N nutrition

Doctoral Thesis/Dissertation

For the attainment of the academic degree of Doctor of Science in Biological Sciences (PhD)

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List of abbreviations

Abbreviation	Meaning
%	percent
°C	degree celcius
µL	micro litre
atom%	atomic percent
$Ca(NO_3)_2x4H_2O$	Calcium nitrate tetrahydrate
$CaCl_2$	Calcium chloride
cDNA	complementary DNA
$CHCl_3$	Chloroform
cm	centimetre
CO_2	Carbon dioxide
$CoCl_2x6H_2O$	Cobalt(II) chloride hexahydrate
$CuCl_2$	Copper(II) chloride
Fac water	Formic acid in water
Fe-EDTA	Iron ethylenediaminetetraacetic acid
g	gramm
GSH	Glutathione
h	hours
H_2O	water
H_2S	Hydrogen sulfide
H_3BO_3	Boric acid
HCl	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
KCl	Potassium chloride
KH_2PO_4	Potassium phosphate monobasic
KI	Potassium iodide
KNO_2	Potassium nitrite
KNO_3	Potassium nitrate
KOH	Potassium hydroxide
L	liter
LiCl	Lithium chloride
MeOH	Methanol
MES	2-Morpholinoethanesulfonic acid
$MgSO_4x7H_2O$	Magnesium sulfate heptahydrate
MilliQ	Millipore Milli-Q lab water
min	minutes
mL	millilitre
mM	millimolar
$MnCl_2x4H_2O$	Manganese(II) chloride tetrahydrate
Na_2CO_3	Sodium carbonate
Na_2MoO_4	Sodium molybdate
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide

NH_4^+	ammonium
NH_4Cl	Ammonium chloride
NO_3^-	Nitrate
NR	Nitrate reductase
OAS	O-Acetylserine
PO_4^{3-}	Phosphate
RNA	Ribonucleic acid
RP-HPLC	Reversed-Phase-High Performance Liquid Chromatography
rpm	revolutions per minute
RT	room temperature
RT-qPCR	Real-time quantitative Polymerase chain reaction
s	seconds
SDS	Sodium dodecyl sulfate
SO_4^{2-}	Sulfate
TRIS	Tris(hydroxymethyl)aminomethan
VPD	vapor pressure deficit
$ZnCl_2$	Zinc chloride

Abstract

The evolution of C_4 photosynthesis led to increased photosynthetic efficiency compared to C_3 relatives particularly in warm and dry conditions, offering great potential to improve crop performance under increasingly arid conditions brought on by climate change. C_4 plants achieve this improved photosynthetic efficiency by employing a CO_2-concentrating mechanism which minimizes the oxygenation reaction of RuBisCO. This mechanism depends on spatial separation of primary CO_2 fixation by PEP carboxylase (PEPC) in mesophyll (MS) cells, transfer of the C_4 acid intermediates to bundle sheath (BS) cells for decarboxylation, and the assimilation of the CO_2 by RuBisCO. Through this compartmentalization the N balance between MS and BS cells is disrupted, nitrate reduction is specifically localized in MS, and C_4 plants possess a higher nitrogen use efficiency. However, so far it is not sufficiently understood how N uptake and assimilation adapted in the course of C_4 photosynthesis evolution. Identifying these alterations in the N assimilation pathway will be necessary to support efforts of engineering the more efficient C_4 photosynthesis mechanism in C_3 staple crops like rice or wheat.

This thesis characterizes and compares mineral nutrition traits of C_3, C_3-C_4 and C_4 species from the *Brassicales* order to determine metabolic differences dependent on photosynthesis type and how they are conserved among species of the same type. The analyses revealed a higher N deficiency tolerance in the C_4 species, which is achieved through a higher uptake ability and accumulation of NO_3^- and amino acids in leaves, which might serve as a N reserve for production of photosynthetic enzymes to upkeep photosynthesis rates under low N conditions. In contrast, intermediate species did not show an increase in N deficiency tolerance implying that improved N deficiency tolerance is dependent on a complete transition to C_4 photosynthesis. Metabolite profiling further revealed significant differences in accumulation patterns of sugars, amino acids and TCA intermediates between the C_3 and C_4 species under N deficiency conditions suggesting a differential regulation of N deficiency responses in C_3 and C_4 species. ^{15}N uptake and gene expression analysis revealed clear differences in the regulation of the uptake and assimilation of NO_3^- and NH_4^+ between the C_3 and C_4 *Cleome* species. The higher sensitivity to NH_4^+ as their sole N source in C_4 plants was shown to be linked to a lower uptake capacity for NH_4^+.

Overall the results suggest a differential regulation of N assimilation and deficiency responses in C_3, C_3-C_4 and C_4 species from the *Brassicales* order and highlight the importance of understanding the metabolic fluxes to improve plant performance.

1. Introduction

Feeding the world's growing population, which is projected to reach 9.7 billion people by 2050, remains one of today's most significant challenges (UN-DESA-PD, 2022). According to recent reports, nearly 282 million people experienced acute hunger and food insecurity in 2023 (FSIN, 2023). Moreover, nutrient deficiencies are estimated to affect over 2 billion people worldwide (Amoroso, 2016). The predicted increase of atmospheric CO_2 concentrations poses the risk of exacerbating this issue as many crops show an increase in yield, but a decrease in both micronutrient (e.g. zinc and iron) and macronutrient content (e.g. nitrogen) when exposed to elevated CO_2 concentrations. This phenomenon is referred to as the carbon dilution effect (Loladze, 2014; C. Zhu *et al.*, 2018; Ujiie *et al.*, 2019). In contrast, in C_4 plants carbon uptake is saturated at ambient CO_2 levels due to their CO_2 concentration mechanism. While, therefore profiting much less from elevated CO_2 than C_3 plants they are also less affected by the carbon dilution effect (Von Caemmerer and Furbank, 2003; Myers *et al.*, 2014). Due to this C_4 crops harbour a great potential in the pursuit of food security in a future environment with elevated CO_2 levels. Their increased photosynthetic efficiency in warm and dry conditions compared to C_3 relatives, further highlights their potential to improve crop performance under increasingly arid conditions brought on by climate change. However, only ~ 3 % of all angiosperms, including the major crops, maize, sugar cane, and sorghum use C_4 photosynthesis (Kellogg, 2013). To access the potential of C_4 crops, efforts are being made to engineer C_4 photosynthesis into C_3 crops e.g. rice.

Irrespective of nutritional quality, production of enough food to meet the increasing demand and thereby ensuring food security requires the agricultural yield to rise 2.4 % per year, which is far above current rates of around 0.9 – 1.6 % per year for main crops such as wheat and maize. Increasing the agricultural land to raise production is not a sustainable solution, as globally 50 % and on some national levels up to 80 % of habitable land is already devoted to agriculture (Ray *et al.*, 2013).

Since agriculture is highly vulnerable to changes in temperature, precipitation, and rising atmospheric CO_2 concentration, global climate change adds another layer to this problem (Wheeler and Von Braun, 2013; Rosenzweig *et al.*, 2014). Recent extreme weather events such as severe floods, storms, droughts, wildfires, as well as pest and disease outbreaks further pose threats to crop yields and therefore food security (FSIN, 2023).

In countries like Germany, in which the cereal production has grown at a faster rate than the population, cereal production per person has increased despite the growing population (Ritchie and Roser, 2013). Many of the current methods used for intensification of the available land i.e. crop irrigation, nitrogen- and phosphate fertilizer usage, and pesticide application are unsustainable since

they negatively affect ecosystems by depleting natural resources and biodiversity. Yield gaps, defined as the difference between potential and actual yield, reveal regions at risk for future yield stagnation. Analysis of yield gaps gives insights in opportunities to improve agricultural production (Gerber *et al.*, 2024). Furthermore they highlight the importance of research to achieve sustainable intensification of agriculture e.g. on nitrate use efficiency (NUE) to improve sustainable nitrogen management and reduce the need for N fertilizers (Cassman and Grassini, 2020; Tamagno *et al.*, 2024).

1.1. Nitrogen fertilization

Global crop production is reliant on increased yields achieved by N fertilizer usage to ensure food security (Erisman et al., 2008; Liang, 2022). Agricultural production is tightly linked to nutrient input, thus improving N availability through application of N fertilizers has greatly contributed to improving crop yields (Tilman et al., 2002; Sinclair and Rufty, 2012). However, less than 50 % of the N supplied as fertilizer is used up by crops while the rest is lost into the environment owing to volatilization, leaching, run-off and denitrification. N losses thereby cause environmental problems such as the release of greenhouse gases, pollution of water bodies, soil acidification and biodiversity reduction (Fageria and Baligar, 2005; Oenema, van Liere and Schoumans, 2005; Billen, Garnier and Lassaletta, 2013; Bodirsky *et al.*, 2014; Martínez-Dalmau, Berbel and Ordóñez-Fernández, 2021). Reduction of N losses and therefore environmental pollution can be achieved through the development of crop varieties with higher NUEs (yield per unit of N available in the soil) which hinges on a comprehensive understanding of the mechanisms for N uptake, assimilation, and remobilization throughout a plant's life cycle (Kant, Bi and Rothstein, 2011).

Nitrogen (N) is an essential element and critical for the biosynthesis of amino acids, which act as building blocks for proteins, nucleotides, chlorophyll and many other essential cellular components (Lea and Ireland, 1999; Krapp, 2015). In higher plants, N is predominantly taken up from the soil in the form of nitrate (NO_3^-) or ammonium (NH_4^-) (Crawford and Forde, 2002; Bloom, 2015). While plants can take up inorganic N in form of both NO_3^- and NH_4^+ their preference for either N source over the other is highly species-specific (A. J. Miller and Cramer, 2005). Many abiotic factors such as pH, temperature and general nutrient availability play a role in which N source is preferred by a plant in any given environment (Britto and Kronzucker, 2013). N availability is largely dependent on the soil's N composition, which is subject to mineralization (ammonification) and nitrification. Mineralization or ammonification describes the conversion of organic N e.g. manure, organic matter and crop residues into the inorganic forms NH_4^+ and NO_3^- by microorganisms (Jansson and Persson, 1982; Stevenson, 1986; Rengel, 2003). Nitrification is the rapid oxidation of NH_4^+ to NO_3^- under non-limiting conditions, which makes NO_3^- the most abundant N source for plants (Liang and MacKenzie, 1994; Kaboneka, Sabbe and Mauromoustakos, 1997). However, in conditions in which nitrification is limited, such as

acidic soils, high aluminium concentrations, dry or waterlogged soils and low temperature, NH_4^+ is the major N source (Smith and Middleton, 1979; Magalhães *et al.*, 1993).

1.2. N uptake and assimilation

Nitrate uptake from the soil is facilitated by nitrate transporters. In the model organism *Arabidopsis thaliana* (*A. thaliana*), nitrate transporters are categorized into two multigene transporter families, the NFP and NRT family, depending on their belonging to the low (LATS) or high (HATS) affinity transport system (Orsel, Krapp and Daniel-Vedele, 2002; Krapp *et al.*, 2014; Léran *et al.*, 2014). Various transporters from both gene families have been shown to play a role in root nitrate uptake from the soil, including four high affinity NRT2 transporters (NRT2.1, NRT2.2, NRT2.4, and NRT2.5) and two NPF transporters (NRT1.1 and NRT1.2) (Tsay *et al.*, 1993; Huang *et al.*, 1999; Filleur *et al.*, 2001; Kiba *et al.*, 2012; Lezhneva *et al.*, 2014). NRT1.1/NFP6.3 (CHL1) was the first nitrate transporter identified in Arabidopsis (Tsay *et al.*, 1993). NRT1.1 is the only dual-affinity nitrate transporter and also functions as a nitrate sensor (Liu, Huang and Tsay, 1999; Ho *et al.*, 2009; Ye, Tian and Jin, 2019). Changes in nitrate concentrations modulate phosphorylation of the Thr101 of NRT1.1 by CIPK23 (CBL-INTERACTING PROTEIN KINASE 23) causing the switch from the low- to the high-affinity nitrate transport systems (Liu, Huang and Tsay, 1999; Parker and Newstead, 2014; Sun et al., 2014). In Arabidopsis *NRT1.1* is expressed in both roots and shoots, playing a role in both nitrate uptake from the soil and root-to-shoot translocation via the xylem (Huang *et al.*, 1996; Léran *et al.*, 2013). Analysis of homologs in rice and maize imply that functions of NRT1.1 are conserved across different species (Hu *et al.*, 2015; Wen *et al.*, 2017). Rice has three NRT1.1 homologs (OsNRT1.1A/B/C), however, OsNRT1.1B was identified as the functional homolog to AtNRT1.1 as it also shows nitrate-inducible expression in the plasma membrane and is involved in nitrate uptake and transport as well as nitrate signalling (Hu *et al.*, 2015; Wei Wang *et al.*, 2018). More recent studies in rice further showed that nitrate also acts as a signalling module and activates both phosphate and nitrate utilization. In the identified mechanism a regulatory module consisting of OsNRT1.1B, OsSPX4 and OsNLP3 links nitrate sensing at the plasma membrane to the downstream nitrate and phosphate responses in the nucleus (Hu *et al.*, 2019).

Similar to NO_3^-, NH_4^+ uptake from the soil is facilitated by a family of ammonium transporters (AMTs), which can be categorized into AMT1 and AMT2 groups (Loqué and Von Wirén, 2004). Number, expression pattern, and function of AMTs vary between plant species (Yuan *et al.*, 2007, 2009; Guether *et al.*, 2009; McDonald, Dietrich and Lutzoni, 2012; Li *et al.*, 2016; Giehl *et al.*, 2017; Song *et al.*, 2017; Y. Zhu *et al.*, 2018). In Arabidopsis, NH_4^+ transport is facilitated by six genes (*AMT1.1, AMT1.2, AMT1.3, AMT1.4, AMT1.5, and AMT2.1*) encoding high-affinity ammonium

transporters (AMTs), whose expression is upregulated by N limitation. In low N conditions, NH_4^+ uptake from the soil is mediated by AMT1s. AMT1;1, AMT1;3, and AMT1;5 are expressed in the root tips and epidermal cells (Loqué *et al.*, 2006; Yuan *et al.*, 2007). AMT2;1, however, is mainly expressed in the pericycle, likely contributing to root-to-shoot transport of NH_4^+ (Giehl *et al.*, 2017; Koltun *et al.*, 2022). High external NH_4^+ concentrations lead to inactivation of AMT1s by phosphorylation of a conserved threonine residue to prevent NH_4^+ accumulation and toxicity (Neuhäuser *et al.*, 2007; Lanquar *et al.*, 2009). Inhibition of AMT1.1 and AMT1.2 by phosphorylation is catalysed by CIPK23, like in the case of NRT1.1, however, the phosphorylation is CBL1-dependent instead of CBL9-dependent (Straub, Ludewig and Neuhäuser, 2017).

NO_3^- can be either directly assimilated in the roots or the shoots or transported to the vacuole for storage. For assimilation in the shoots, nitrate is transported via the xylem, a process involving various NRTs including the low-affinity pH-dependent bidirectional nitrate transporter NPF7.3 (NRT1.5), which is expressed in pericycle cells and was shown to be necessary to load nitrate into the xylem (Lin *et al.*, 2008). However, other nitrate transporters including NPF6.3 (NRT1.1) and NPF6.2 (NRT1.4) might also contribute to root-to-shoot transport of nitrate (Léran *et al.*, 2013; Krapp *et al.*, 2014). The reduction of NO_3^- to NO_2^- is catalyzed by the nitrate reductase (NR), a cytosolic enzyme which requires NAD(P)H as a reducing agent (Figure 1) (Campbell, 1999, 2001).

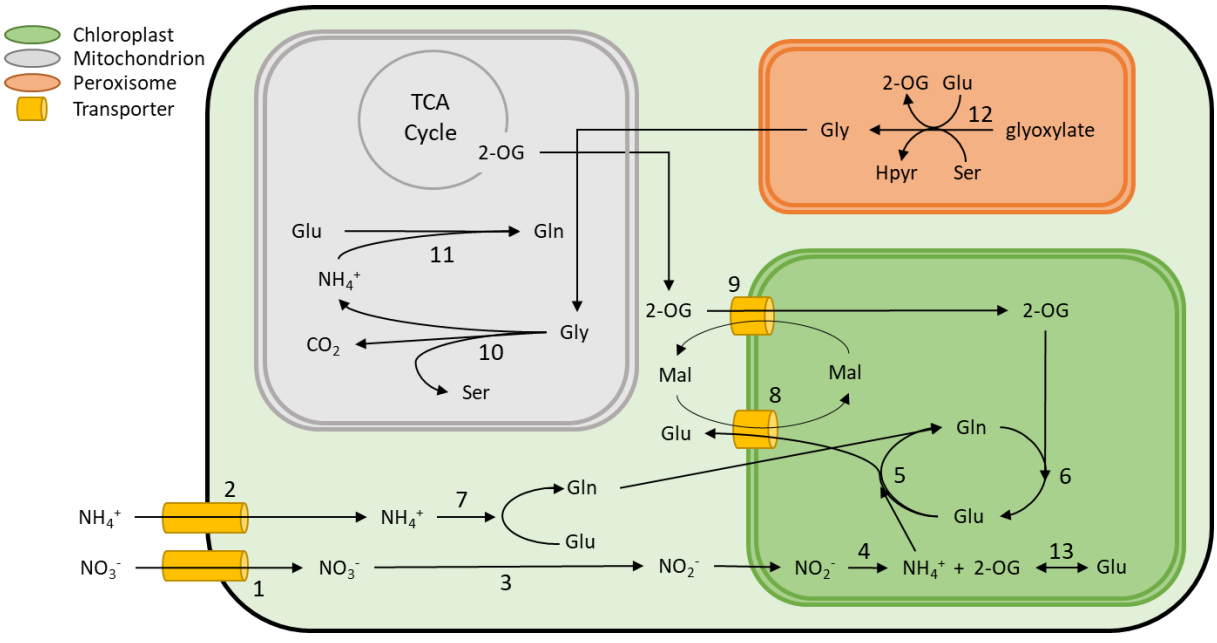

Figure 1: Schematic representation of plant nitrate assimilation. Enzymes and transporters are symbolized by numbers: 1 - nitrate transporter (NRT); 2 - ammonium transporter (AMT); 3 - nitrate reductase (NR); 4 - nitrite reductase (NiR); 5 - plastidic glutamine synthase (GS); 6 - glutamate synthase (GOGAT); 7 - cytosolic (GS); 8 - plastidic glutamate-malate translocator; 9 - plastidic 2-oxoglutarate-malate translocator; 10 - glycine decarboxylase (GDC)/serine hydroxymethyl transferase (SHMT); 11 - mitochondrial GS; 12 - serine glyoxylate aminotransferase (SGAT); 13 - glutamate dehydrogenase (GDH).

Nitrite accumulation is toxic to most plants, therefore nitrite is quickly transported into the plastids, where it is reduced to NH_4^+ by nitrite reductase (NiR) using ferredoxin reduced in the electron transfer chain, thus further linking N assimilation to photosynthesis (Rathnam and Edwards, 1976). The glutamine synthase (GS) enzyme catalyzes the ATP-dependent fixation of ammonium from various sources, including photorespiration, to the δ-carboxyl group of glutamate (Glu) to form glutamine (Gln). Glu for ammonium assimilation is provided by glutamate synthase (GOGAT) which catalyzes the conversion of Gln and 2-oxoglutarate (2-OG) to two molecules of Glu. The additional Glu molecule per GOGAT cycle can then be used to transfer the assimilated N into other amino acids through reactions catalyzed by aminotransferases or transaminases (Forde and Lea, 2007; Bernard and Habash, 2009). Since synthesis of Gln and Glu, beside C-skeletons also requires energy (ATP) and reducing power (NADH), joined regulation of C and N metabolism is necessary for amino acid biosynthesis. The coordination of C and N assimilation requires a complex signalling network integrating N availability with environmental factors impacting photosynthetic efficiency and photorespiration rates, e.g., CO_2 concentration and light conditions (Huppe and Turpin, 1994; Foyer, Ferrario-Méry and Noctor, 2001; Lancien, Gadal and Hodges, 2002; Stitt *et al.*, 2002; Britto and Kronzucker, 2005).

1.3. Photorespiration

Due to its dual-specificity for O_2 and CO_2, ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) catalyzes both the carboxylation and the oxygenation of ribulose 1,5-bisphosphate (RuBP), producing 3-phosphoglyceric acid (3PGA) and 2-phosphoglycolate (2PG), respectively (Ogren, 1984). Unlike 3PGA, 2PG cannot enter the Calvin-Benson-Bassham-Cycle (CBBC) and instead must be converted to 3PGA via photorespiration (Leegood *et al.*, 1995). The photorespiration pathway spans across three organelles: the chloroplast, the peroxisome, and the mitochondrion. As a first step, 2PG is decarboxylated to glycolate by 2-phosphoglycerate phosphatase (PGLP) (Figure 2).

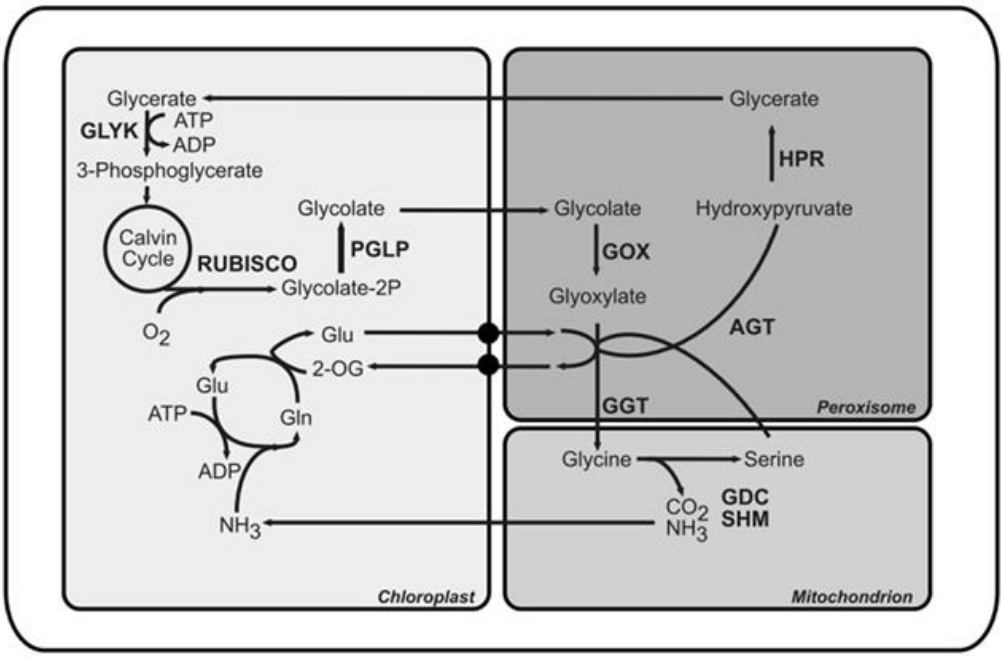

Figure 2: Schematic description of the photorespiratory pathway. Abbreviations: AGT: serine glyoxylate aminotransferase; GDC: glycine decarboxylase complex; GGT: glutamate, glyoxylate-aminotransferase; GLYK: D-glycerate 3-kinase; GOX: glycolate oxidase; HPR: hydroxypyruvate reductase; PGLP: 2-phosphoglycerate phosphatase; SHM: serine hydroxymethyltransferase; RUBISCO: Ribulose-1,5-bisphosphate-carboxylase/-oxygenase; 2-OG: oxoglutarate; 3-PGA: 3-phosphoglycerate; Gln: glutamine; Glu: glutamate (Mallmann *et al.*, 2014).

After being transported to the peroxisome, glycolate is first oxidized to glyoxylate and subsequently transaminated producing the amino acid glycine (Gly). In the mitochondria, the produced Gly is rapidly converted to serine (Ser) by the Gly decarboxylase (GDC)/Ser hydroxymethyltransferase (SHM) enzyme complex, making photorespiration, next to glycolysis and primary N assimilation, an important source of serine in C_3 plants. Serine has to be transported back to the peroxisome, where it is deaminated by the serine:glyoxylate aminotransferase (SGAT) to generate hydroxypyruvate. The enzyme hydroxypyruvate reductase1 (HPR1) reduces hydroxypyruvate to glycerate, which is transported to the chloroplast for phosphorylation. Glycerate kinase (GLYK) catalyses the final step of the photorespiratory pathway producing 3PGA, which can re-enter the CBBC (Bauwe, Hagemann and Fernie, 2010; Bauwe, 2011). The decarboxylation of Gly by the GDC/SHM complex also releases CO_2 and NH_3 (Bauwe, Hagemann and Fernie, 2010; Bauwe, 2011). The photorespiratory NH_4^+ has to be re-assimilated by glutamine synthase (GS) and the ferredoxin-dependent glutamine oxoglutarate aminotransferase (GOGAT) in the chloroplast (Keys *et al.*, 1978; Coschigano *et al.*, 1998). The re-assimilation of NH_4^+ into glutamate requires 2-oxoglutarate (2OG), which is imported by a 2-oxoglutarate/malate transporter (Kinoshita *et al.*, 2011). Dit2.1, a glutamate/malate transporter, facilitates the export of the produced malate to the cytosol (Renné *et al.*, 2003). Glutamate can then act as an amino group donor for peroxisomal conversion of glyoxylate to glycine by GGT (Liepman and Olsen, 2003). Photorespiration is an energetically-expensive process which consumes ATP and

NAD(P)H and as a result reduces the CO_2 assimilation efficiency and biomass production. Under unfavourable environmental conditions, i.e. low atmospheric CO_2 and high temperatures, photorespiration can lead to yield losses of 30 to 50 % in C_3 plants (Sage, 2001, 2013; Bauwe, Hagemann and Fernie, 2010; Raines, 2011).

1.4. C_2 photosynthesis

C_3C_4 intermediate plants, first described by Kennedy et al. in 1974, exhibit intermediate CO_2 compensation points relative to C_3 and C_4 plants (Kennedy and Laetsch, 1974; Monson et al., 2000). The underlying reduction in photorespiration in C_3C_4 intermediates is achieved by operating a photorespiration-driven carbon-concentrating mechanism (CCM), the so-called C_2 cycle or glycine shuttle (Rawsthorne, 1992; Monson et al., 2000). Loss of GDC activity in the MS cells leads to the restriction of photorespiration to BS cells, therefore Gly produced in MS has to be transported to the BS cells, where decarboxylation of said Gly leads to a higher CO_2 concentration within BS cells (Hylton *et al.*, 1988; Rawsthorne *et al.*, 1988; Rawsthorne and Hylton, 1991; Keerberg *et al.*, 2014). The C_2 cycle allows to capture a large part of CO_2 released during the decarboxylation of glycine and thereby reduces loss of CO_2 through photorespiration. The shift of glycine decarboxylase (GDC) expression from MS to BS cells characteristic for C_3C_4 intermediates is thought to be the first metabolic change towards the evolution of C_4 photosynthesis as it offered an advantage in the evolutionary low CO_2 conditions (Keerberg *et al.*, 2014; Lundgren, 2020). A flux balance analysis performed by Mallmann et al. suggests that the disruption of the N balance between MS and BS cells caused by the establishment of C_2 photosynthesis triggers an ammonia recycling mechanism, which encompasses expression changes of the same genes that are required for evolution of C_4 photosynthesis, indicating that the C_2 cycle might represent a pre-adaptation for the C_4 photosynthesis (Mallmann *et al.*, 2014). However, the analysis of gene regulatory networks (GRNs) of closely evolutionarily related *Flaveria* species identified an alternative for restructuring of N metabolism in the metabolic pathway of the type II C_3-C_4 species *F. ramosissima.* This alternative evolutionary solution to the ammonia imbalance could allow the co-evolution of the stable state C_2 species parallel to C_4 species (Amy Lyu *et al.*, 2023). The resulting C_2 species could, additionally to their higher resource use efficiency, offer a broader environmental range compared to most C_4 species (Walsh *et al.*, 2023).

1.5. C_4 photosynthesis

In C_4 photosynthetic plants the oxygenation reaction of RuBisCO is reduced through implementation of a carbon concentrating mechanism (CCM), which increases the CO_2 concentration around the enzyme, resulting in improved photosynthetic efficiency and decreased yield losses due to photorespiration (Slack, Hatch and Goodchild, 1969). To achieve this, in C_4 plants carbon assimilation

is divided into two steps partitioned between two distinct cell types, the mesophyll (MS) and bundle-sheath (BS) cells, forming the characteristic Kranz anatomy (Dengler and Nelson, 1999). Besides changes in leaf anatomy, strict spatial separation of the required enzymes is necessary to enable the C_4 mechanism of CO_2 fixation. (Sage, Sage and Kocacinar, 2012). In C_4 plants, CO_2 is converted to bicarbonate (HCO_3^-) catalyzed by carbonic anhydrase (CA) in MS cells (Bräutigam *et al.*, 2011). Subsequently, HCO_3^- is fixed to 2-phosphoenolpyruvate (PEP) by PEP carboxylase (PEPC) producing the C_4-acid oxaloacetate (OAA) which is transaminated and transported to the BS cells to be decarboxylated and release the CO_2 (Hatch, Kagawa and Craig, 1975; Hatch, 1987; Sage, 2004) (Figure 3).

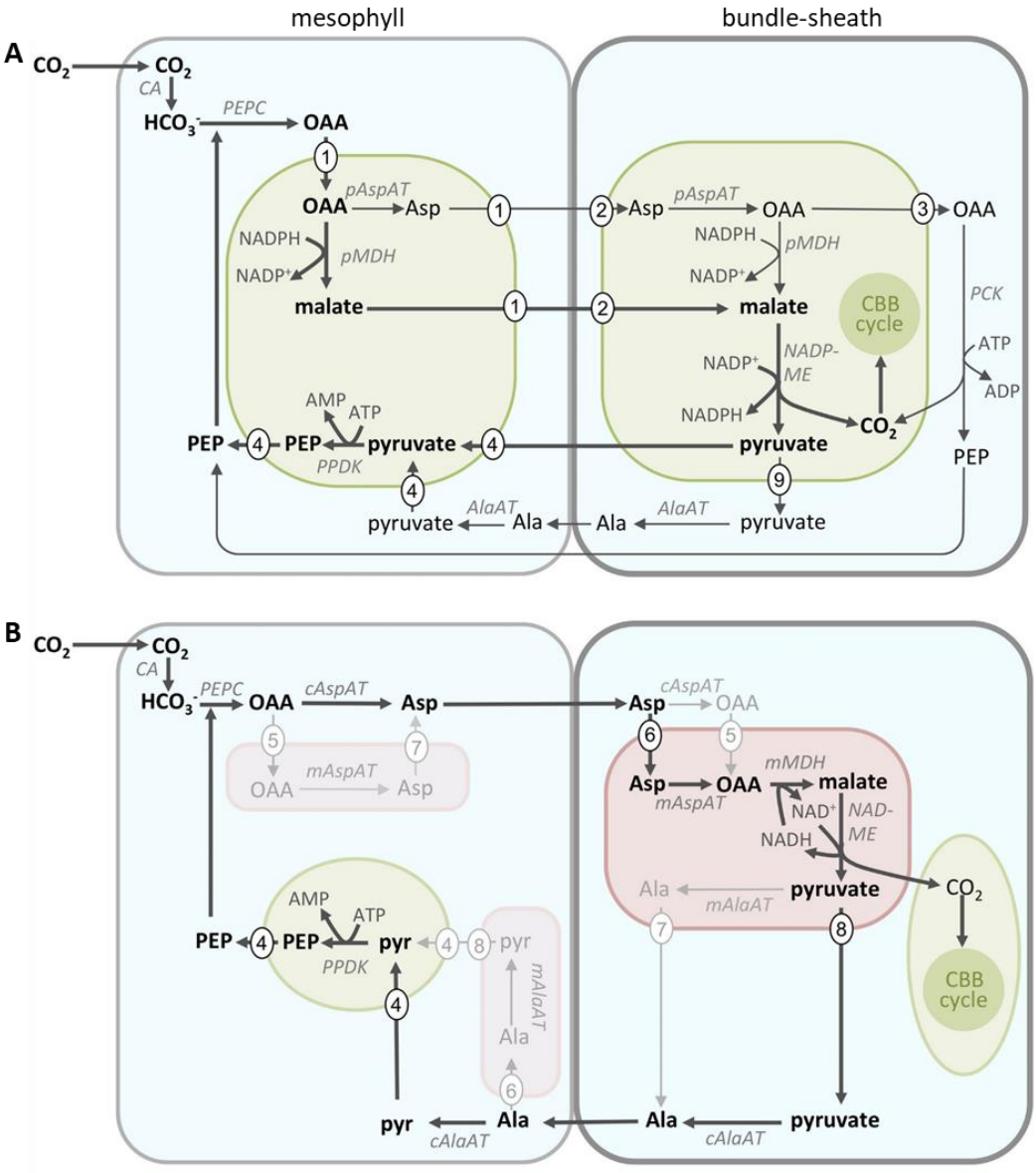

Figure 3: Schematic description of the (A) NADP-malic enzyme and (B) NAD-malic enzyme photosynthetic pathway. Abbreviations of participation enzymes and metabolites: CA - carbonic anhydrase; PEPC - phosphoenolpyruvate carboxylase; pMDH - plastidial NADP-dependent malate dehydrogenase; mMDH - mitochondrial NAD-dependent malate dehydrogenase; pAspAT - plastidial Asp aminotransferase; cAspAT -

cytosolic Asp aminotransferase; mAspAT - mitochondrial Asp aminotransferase; AlaAT - Ala aminotransferase; PCK - phosphoenolpyruvate carboxykinase; NADP-ME - NADP-dependent malic enzyme; NAD-ME - NAD-dependent malic enzyme; PPDK - pyruvate Pi dikinase; HCO_3^- - bicarbonate; OAA – oxaloacetate; Asp – aspartate; Ala - alanine; PEP – phosphoenolpyruvate. Numbers symbolizing transporters: (1) plastidial exchange malate/Asp vs OAA (DIT1/DIT2); (2) plastidial malate/Asp exchange (DCT2), (3) an unknown plastidial OAA exporter; (4) plastidial pyruvate/PEP exchanger (BASS2/NHD/PPT or an unknown transporter; (5) mitochondrial dicarboxylate exchanger; (6) unknown mitochondrial amino acid importer; (7) unknown mitochondrial exporter; (8) unknown mitochondrial pyruvate exporter; (9) unknown plastidial pyruvate exporter. The light grey reactions indicate possible pathways in *C. gynandra* assuming that all C_4-specific Ala ATs and Asp ATs are indeed localised to the mitochondria of mesophyll and bundles sheath cells as described by (Schlüter *et al.*, 2019).

The assimilation steps following the production of OAA vary between biochemical subtypes which are categorized according to the enzyme catalysing the decarboxylation reaction, i.e. phosphoenolpyruvate carboxykinase (PCK), NADP malic enzyme (NADP-ME) or NAD malic enzyme (NAD-ME). However, contrary to this strict categorisation, many NAD-ME and NADP-ME C_4 species have been shown to also utilize PEPCK-based decarboxylation as well (Furbank, 2011; Pick et al., 2011; Wang et al., 2014).

In the NADP-ME subtype, malate is formed from OAA in the chloroplast catalyzed by NADP-dependent malate dehydrogenase (pMDH) before being transported to BS cells. BS-specific malate decarboxylation by the NADP-ME enzyme leads to the release of CO_2, NADPH, and pyruvate. RuBisCO localized in the BS cells re-assimilates the released CO_2 into the Calvin-Benson-Bassham Cycle (CBBC). The pyruvate is transported back to the MS, where it is recycled to PEP via phosphorylation by pyruvate phosphate dikinase (PPDK) using two molecules of ATP. In contrast, in NAD-ME plants aspartate is synthesized from OAA in the MS cytosol and transported to the BS mitochondria, where it might be converted to OAA and subsequently malate catalyzed by Asp aminotransferase (AspAT) and NAD-dependent malate dehydrogenase (MDH). Pyruvate formed by the NAD-ME decarboxylation reaction in the mitochondria, is partially transaminated into alanine (Ala) by Ala aminotransferase (AlaAT), transported to MS cells and converted back to pyruvate for PEP regeneration in MS cell chloroplasts (Hatch, 1987; Weber and von Caemmerer, 2010; Ludwig, 2016). However, aspartate has also been shown to contribute CO_2 release in the BS cells in the NADP-ME type species *Z. mays* and *F. bidentis* (Meister, Agostino and Hatch, 1996). Analyses of transcriptome and proteome data suggest that lower aspartate levels may reduce protein synthesis rates under N deficiency, which could explain higher nitrogen use efficiency (NUE) of NADP-ME relative to NAD-ME species (Khamis, Lamaze and Farineau, 1992; Majeran *et al.*, 2005; Bräutigam *et al.*, 2011; Gowik and Westhoff, 2011; Bräutigam and Gowik, 2016). Comparative studies of C_3 and C_4 species of the same genus have shown that the CCM of the C_4 species enhances their CO_2 assimilation rate under ambient CO_2 and high light conditions leading to an increase in both photosynthetic nitrogen use efficiency (PNUE) and leaf water use efficiency (WUE) compared to C_3 species (Bolton and Brown, 1980; Monson, 1989)(Monson, 1989,Bolton and Brown, 1980).

1.6. Mineral nutrition in C_4 plants

In various C_4 species, 90 % of the activity of the first two enzymes in the S assimilation pathway, ATP sulfurylase (ATPS) and APS reductase (APR), is confined to BS cells (Gerwick, Ku and Black, 1980; Schmutz and Brunold, 1984; Kopriva and Koprivova, 2005; Kopriva, 2011). Furthermore, while showing no differences in the spatial distribution of APR between MS and BS cells, C_4 *Flaveria* species accumulated higher amounts of cysteine (Cys) and glutathione (GSH) compared to their C_3 counterparts (Koprivova *et al.*, 2001). A comparative analysis of *Flaveria* species with different photosynthetic properties in nutrient-controlled environments revealed that C_4 *Flaveria* species accumulate more Cys and GSH than C_3 species under both normal and low S supply, demonstrating a connection between C_4 photosynthesis and alterations in S homeostasis in the *Flaveria* genus (Gerlich *et al.*, 2018a).

Like N, sulfur (S) is essential for plant growth due to roles in cell structure and metabolism. S is taken up from the soil in the form of sulfate by most plants and assimilated into the amino acids cysteine (Cys) and methionine (Met) making it an important constituent of proteins. Due to its inert nature, sulfate requires activation by ATP sulfurylase (ATPS). ATPS catylzes the transfer of sulfate onto an α-phosphate residue of ATP resulting in adenosine-5'-phosphosulfate (APS). APS is then either reduced by APS reductase (APR) forming sulfite or phosphorylated by APS kinase to producing 3'-phosphoadenosine 5'-phosphosulfate (PAPS), a universal sulfur donor for sulfotransferases. The further reduction of sulfite to sulfide is catalysed by the ferredoxin-dependent sulfite reductase (SIR). Sulfide can be incorporated into the amino acid backbone of O-acetyl-Ser (OAS) by OAS (thiol)lyase (OAS-TL) forming Cys (Takahashi et al., 2011). Cys can either be used in Met biosynthesis or be further incorporated into amino acids, proteins and peptides e.g. GSH (Rouhier, Lemaire and Jacquot, 2008).

OAS is synthesized from serine (Ser) catlyzed by the serine acetyltransferase enzyme (SAT) connecting S metabolism to both photosynthesis and photorespiration but also N metabolism since Ser can be produced via photorespiration, glycolysis, and primary N assimilation (Takahashi et al., 2011)(Figure 4).

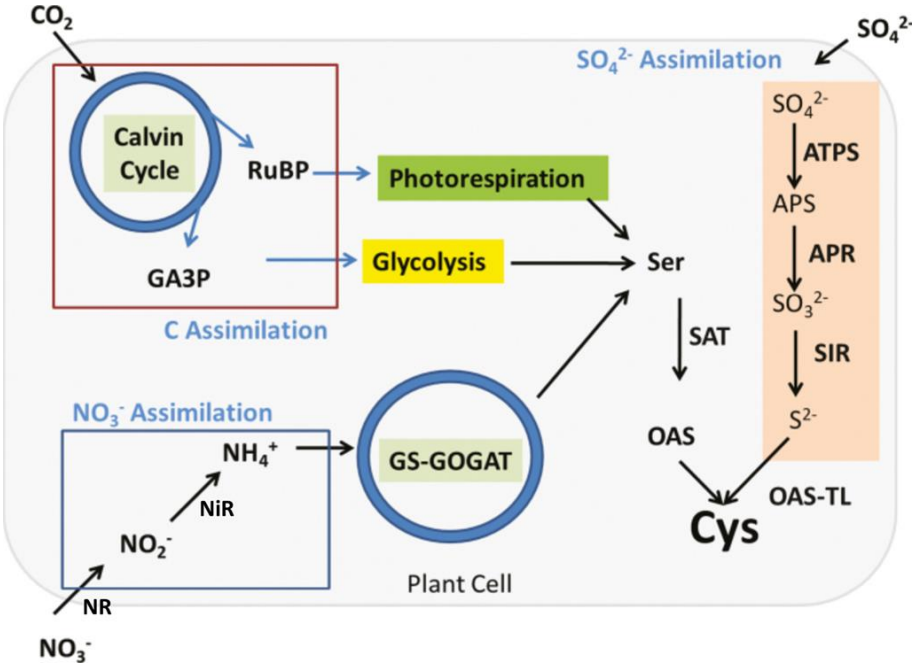

Figure 4: Simplified schematic description of the interconnection of the assimilation of carbon (C) and the major mineral nutrients nitrogen (N) and sulfur (S). Abbreviations: ATPS: ATP sulfurylase; APS: adenosine-5'-phosphosulfate; APR: APS reductase; SIR: sulfite reductase; SAT: Ser acetyltransferase; OAS: O-acetyl-Ser; OAS-TL: OAS (thiol)lyase; GA3P: 3-phosphoglyceric acid; RuBP: ribulose-1,5-bisphosphate, NR: nitrate reductase, NiR: nitrite reductase. Figure modified from (Jobe *et al.*, 2019).

In C_4 plants the reduction of nitrate and nitrite is restricted to MS cells, while the further assimilation of reduced nitrogen into the amino acids glutamate and glutamine takes place in the BS or both MS and BS cells (Rathnam and Edwards, 1976; Moore and Black Jr., 1979; Becker, Carrayol and Hirel, 2000). Photorespiration and therefore production of serine and respiratory NH_4^+ are restricted to BS cells (Hylton et al., 1988; Morgan, Turner and Rawsthorne, 1993). However, respiratory NH_4^+ is recycled via the photorespiratory nitrogen cycle in the mesophyll cells. Therefore, to maintain homeostasis of both carbon and nitrogen metabolic pathways have to be adjusted (Keys et al., 1978; Monson, Rawsthorne and Centre, 2000; Keys, 2006). The resulting alterations in N metabolism and their effect on the regulation of N uptake and assimilation are so far not sufficiently understood.

C_4 photosynthesis is a complex trait that has evolved independently more than 60 times in at least 19 families including both monocot and dicot species, which implies a low evolutionary barrier and has therefore raised interest in introducing the C_4 mechanism into C_3 crops such as rice (Sage, 2004; Hibberd, Sheehy and Langdale, 2008; Sage, Christin and Edwards, 2011). However, to achieve this goal a deeper understanding of the C_4 metabolism is required, as not only the C metabolism but also the assimilation of the major mineral nutrients N and S will be affected by the engineering of C_4 photosynthesis into C_3 crops.

2. Aims of this thesis

Coordination of carbon (C) and N metabolism is necessary to produce amino acids, proteins, nucleotides, chlorophyll and many other essential cellular components. As part of C_4 evolution the reduction of nitrate and nitrite mainly takes place in MS cells, while photorespiration is restricted to BS cells. This metabolic compartmentalisation disrupts the N balance between MS and BS cells and requires adjustments of the nitrogen metabolic pathways. However, so far it is not sufficiently understood how these alterations affect mineral nutrition and specifically N uptake and assimilation in C_4 plants. As a consequence of their CCM, many C_4 species have an increased photosynthetic NUE efficiency compared to C_3 species and require less RuBisCO (Sage, 2004; Ghannoum *et al.*, 2011). Due to these differences in their N requirement we want to test whether C_4 species are less sensitive to N deficiency conditions, i.e., that they have a higher N deficiency tolerance than their C_3 relatives and potentially C_3-C_4 intermediate species.

1. The first aim of this thesis is to determine whether the C_4 species *C. gynandra* has a higher N deficiency tolerance compared to its C_3 relative *C. hassleriana* and similarly, if the C_3-C_4 intermediate species from the *Brassicaceae* family are more tolerant than closely related C_3 species.

While most plants can take up inorganic N in the form of nitrate (NO_3^-) and ammonia (NH_4^+) (A. J. Miller and Cramer, 2005), the preference for either of these N sources differs widely between species. However, NH_4^+ as a sole N source is known to inhibit photosynthesis in both C_3 and C_4 plants. The CO_2 concentration mechanism inherent to C_4 photosynthesis reduces the oxygenation rate of RuBisCO and therefore, the rate of photorespiration. The reduced photorespiratory flux lowers the amount of released photorespiratory NH_4^+, meaning C_4 species might be less adapted to dealing with high NH_4^+ levels.

2. Thus, this thesis aims to characterise the effect of NH_4^+ supplementation on the photosynthetic efficiency and biomass production of C_3, C_3-C_4 and C_4 *Brassicales* species to answer whether C_4 evolution leads to an increased sensitivity to NH_4^+ toxicity.
3. After characterizing the differential effects of N deficiency and NH_4^+ toxicity, this study further aims to elucidate alterations in the mechanism underlying the regulation of N uptake and assimilation by N deficiency and different N sources in C_4 species compared to a close C_3 relative.

Overall this project aims to identify differences in N assimilation and N deficiency responses in C_3, C_3-C_4 and C_4 species from the *Brassicales* order by characterizing mineral nutrition traits and determining to what extent these metabolic differences are conserved within species with the same photosynthesis type. Identifying these alterations in the N assimilation pathway, that are consequences of or even prerequisites for C_4 evolution, will be crucial to support efforts of engineering the more efficient C_4 photosynthesis mechanism in C_3 staple crops like rice or wheat.

3. Methods and materials

3.1. Plant material

For this study a panel of closely related C_3, C_3C_4-intermediate and C_4 *Brassicales* species previously established and characterized by Schlueter *et al.* was used (Table 1). This panel included the C_3 species *Cleome hassleriana* (*Tarenya hassleriana*) and its closest C_4-relative *Cleome gynandra* (*Gynandropsis gynandra*). The study further included C_3 species (*Diplotaxis viminea*, *Moricandia moricandioides*) and C_3C_4-intermediate species (*Diplotaxis muralis*, *Diplotaxis tenuifolia*, *Moricandia suffruticosa*, *Moricandia arvensis*) from the *Diplotaxis* and *Moricandia* genera (Schlüter *et al.*, 2017).

Table 1: *Brassicales* species used in this study. Table lists the species used in this study with their corresponding photosynthesis type and CO_2 compensation point.

Species	**CO_2 compensation point**	**type**
C. hassleriana	62.5	C_3
M. moricandioides	51.586557	C_3
D. viminea	51.083105	C_3
D. muralis	35.040164	C_3- C_4*
M. suffruticosa	24.872566	C_3- C_4
M. arvensis	24.424612	C_3- C_4
D. tenuifolia	12.681964	C_3- C_4
C. gynandra	4.278226	C_4

3.2. Plant cultivation

Seeds were germinated in standard soil. Two weeks after germination the seedlings were transferred to pots (Ø 6 cm) containing a vermiculite-sand-mixture (2:1). For treatment plants were bottom watered with Hoagland nutrient solution with different nitrogen sources for 2-3 weeks depending on the experiment (Table 2+3).

Table 2: Composition of full and limiting Hoagland solution. Hoagland medium was prepared from stock solutions. For N deficiency treatments $Ca(NO_3)_2x4H_2O$ and KNO_3 were either completely or partially replaced with $CaCl_2$ and KCl, respectively. For experiments including NH_4^+ as an alternative N source both $Ca(NO_3)_2x4H_2O$ and KNO_3 were either completely or partially replaced with NH_4Cl (see Table 3).

Macroelements	**Final conc.**	**Stock conc.**	**Amount/L**
$Ca(NO_3)_2x4H_2O$ *, ***	1.5 mM	141.7 g/L	2.5 mL
KNO_3 **, ***	1 mM	40.4 g/L	2.5 mL
KH_2PO_4	0.75 mM	40.8 g/L	2.5 mL
$MgSO_4x7H_2O$	0.75 mM	74 g/L	2.5 mL
Fe-EDTA	0.1 mM	14.7 g/L	2.5 mL
Microelements			
$MnCl_2x4H_2O$	10 µM	1.98 g/L	1 mL
H_3BO_3	50 µM	3.1 g/L	1 mL
$ZnCl_2$	1.75 µM	238 mg/L	1 mL
$CuCl_2$	0.5 µM	67.2 mg/L	1 mL
Na_2MoO_4	0.8 µM	164.7 mg/L	1 mL
KI	1 µM	166 mg/L	1 mL
$CoCl_2x6H_2O$	0.1 µM	23.8 mg/L	1 mL
Substitutions for deficiency treatments			
* $CaCl_2$	varied depending on experiment	66.6 g/L	2.5 mL
** KCl		22.36 g/L	2.5 mL
*** NH_4Cl		85.58 g/L	2.5 mL

Table 3: Composition of Hoagland media containing both nitrate and ammonium. Amounts of KH_2PO_4, $MgSO_4x7H_2O$, Fe-EDTA and all micronutrients remain unchanged between treatments.

Final N conc	**Macroelements**	**Amount/L**
N deficency treatments		
4 mM	$Ca(NO_3)_2x4H_2O$ KNO_3	2.5 mL 2.5 mL
1 mM	KNO_3 $CaCl_2$	2.5 mL 2.5 mL
0.5 mM	KNO_3 $CaCl_2$ KCl	1.25 mL 2.5 mL 1.25 mL
0.25 mM	KNO_3 $CaCl_2$ KCl	0.625 mL 2.5 mL 1.875 mL
NH_4^+ treatments		

0 mM NH_4^+/ 4 mM NO_3^- (0 %)	$Ca(NO_3)_2x4H_2O$ KNO_3	2.5 mL 2.5 mL
0.4 mM NH_4^+/ 3.6 mM NO_3^- (10 %)	$Ca(NO_3)_2x4H_2O$ KNO_3 $CaCl_2$ NH_4Cl	2.167 mL 2.5 mL 0.333 mL 0.25 mL
1 mM NH_4^+/ 3 mM NO_3^- (25 %)	$Ca(NO_3)_2x4H_2O$ KNO_3 $CaCl_2$ NH_4Cl	1.667 mL 2.5 mL 0.834 mL 0.625 mL
2 mM NH_4^+/ 2 mM NO_3^- (50 %)	$Ca(NO_3)_2x4H_2O$ KNO_3 $CaCl_2$ NH_4Cl	0.833 mL 2.5 mL 1.667 mL 1.25 mL
3 mM NH_4^+/ 1 mM NO_3^- (75 %)	KNO_3 $CaCl_2$ NH_4Cl	2.5 mL 2.5 mL 0.25 mL
3.5 mM NH_4^+/ 0.5 mM NO_3^- (87.5 %)	KNO_3 $CaCl_2$ NH_4Cl KCl	1.25 mL 2.5 mL 2.187 mL 1.25 mL
3.6 mM NH_4^+/ 0.4 mM NO_3^- (90 %)	KNO_3 $CaCl_2$ NH_4Cl KCl	1 mL 2.5 mL 2.25 mL 1.5 mL
4 mM NH_4^+/ 0 mM NO_3^- (100 %)	$CaCl_2$ NH_4Cl KCl	2.5 mL 2.5 mL 2.5 mL

3.2.1. N re-supply

Seeds were germinated in standard soil. Two weeks after germination the seedlings were transferred to pots (Ø 6 cm) containing a vermiculite-sand-mixture (2:1). Plants were then bottom watered with Hoagland nutrient solution containing 1 mM nitrate for 2 weeks (Table 2).

After 2 weeks the plants were transferred to trays containing nutrient solution with either 4 mM NO_3^- (0 %) or 4 mM NH_4^+ (100 %) nutrient solution (s. Table 3). Samples were collected in 1 mL reaction tubes containing 3 glass beads 0.5, 1, 4 and 24 h after the transfer to the ^{15}N-labelled nutrient solution and immediately flash frozen in liquid nitrogen.

3.2.2. ^{15}N feeding

Seeds were germinated in standard soil. Two weeks after germination the seedlings were transferred to pots (Ø 6 cm) containing a vermiculite-sand-mixture (2:1). Plants were then bottom watered with Hoagland nutrient solution containing 1 mM nitrate for 2 weeks (Table 2).

After 2 weeks the plants were transferred to trays containing nutrient solution with either 4 mM of 98 atom% $K^{15}NO_3$ or $^{15}NH_4Cl$, respectively. Samples were collected in 2 mL reaction tubes containing 3 glass beads 4, 8, 24 and 48 h after the transfer to the ^{15}N-labelled nutrient solution and immediately flash frozen in liquid nitrogen.

3.3. Quantification of CO_2 assimilation using infra-red gas exchange analysis (IRGA)

CO_2 is an heteroatomic molecule and absorbs infra-red radiation at a wavelength of 4.25 mm. CO_2 assimilation was measured as the difference in CO_2 absorption using the LI-6800 infra-red gas exchange analyser (LI-6800, LiCor Biosciences, Lincoln, NE, USA). Plant leaves were enclosed in a sample chamber under controlled conditions (Flowrate: 400 µmol s^{-1}; H_2O: VPD 1.5 kPa; CO_2: 400 µmol mol^{-1}; Fan: 10000 rpm; Temp.: 22-25 °C; Light: 1500). CO_2 absorption was measured at 13 different CO_2 concentrations (10, 50, 100, 200, 250, 300, 400, 500, 600, 800, 1000, 1250, 1500 ppm). In the case of the C_4 species, additional CO_2 concentration (20, 30, 70 ppm) were included in the measurement. CO_2 assimilation rate "A" and internal CO_2 concentration "C_i" were calculated using the following formulas:

$$A = \frac{Flow \cdot 1 \cdot \left(CO_2r - CO_2s \cdot \frac{1000 - 1 \cdot H_2Or}{1000 - 1 \cdot H_2Os}\right)}{100 \cdot LeafArea}$$

$$C_i = \frac{\left(g_{tc} - \frac{E}{2}\right) \cdot C_a - A}{g_{tc} + \frac{E}{2}}$$

CO_2 assimilation "A" was then plotted against the respective internal CO_2 concentration "C_i" to produce A-Ci-curves depicting the plants' response to varying CO_2 conditions. Using only values from the linear section of the CO_2 assimilation curve, CO_2 compensations points were calculated as intersects with the x-axis.

3.4. Nitrate reductase activity

For the determination of nitrate reductase (NR) enzyme activity about 50 mg shoot or root material was extracted in 500 µL of extraction buffer (100 mM KH_2PO_4 pH 7.5, 1 mM EDTA). The extracts were centrifuge at max. speed for 15 min at 4 °C. 200 µL of the resulting supernatant was combined with 50 µL 100 mM KNO_3 and 250 µL 100 mM KH_2PO_4. After adding 100 µL 2 mM NADH, the samples were incubated for 30 min at 30 °C. For quantification of the produced nitrite 250 µL 1 % sulphanilamide and 0.02 % N-(1-Naphthyl)ethylenediamine (NED) were added to the reaction tube. After incubation for 30 min absorbance was measured at 540 nm. For calibration, a serial dilution of KNO_2 (0.025, 0.05,

0.1, 0.2, 0.4 mM) was used. NR activity was normalized by dividing it with the concentration of total soluble protein content (see 3.5.2).

3.5. Metabolite Analysis

3.5.1. Isolation and quantification of anions

For quantification of anion content frozen plant material was homogenized in 1000 µL H_2O. The extracts were shaken at 4 °C for 1 h and subsequently incubated at 95 °C for 15 min. After centrifugation at max. speed and 4 °C for 15 min 100 µL of the supernatant was transferred into glass vials and diluted 1:10 with H_2O. The amounts of various anions were quantified using a Dionex ICS-1100 chromatography system with a Dionex IonPac AS22 RFIC 4c 250 mm analytical column (Thermo Scientific, Darmstadt, Germany) as stationary phase and a 4.5 mM Na_2CO_3/1.4 mM $NaHCO_3$ buffer as mobile phase (Huang *et al.* 2016).

3.5.2. Isolation and quantification of soluble proteins

Frozen plant tissue was homogenized and extracted in 1000 µL 0.1 mM NaOH (pH 12.8). After a 30 min incubation at RT the samples were centrifuged for 5 min at max. speed. Protein concentration was measured using the Bradford method (Bradford, 1976). For analysis, 10 µL of the supernatant was transferred into new reaction tubes, diluted with 790 µL water and mixed with 200 µL Bradford reagent dye (BioRad; USA). Following a 15 min incubation at RT, absorbance at 595 nm was measured using an InfinitePro 200 TECAN reader (Tecan, Switzerland). External standards of bovine serum albumin (2.5, 5, 7.5, 10 µg/mL) were used to generate a standard curve.

3.5.3. Metabolic profiling (GC-MS)

Plant tissue was collected in 2 mL reaction tubes containing 3 glass beads and immediately flash frozen in liquid nitrogen. An extraction mixture containing H_2O:MeOH:$CHCl_3$ in a ratio of 1:2.5:1 was prepared and precooled to -20 °C overnight. As an internal standard 5mM Ribitol/DMPA stock solution was added to the extraction mixture. After adding 500 µL of the extraction mixture, the frozen samples were homogenized with an Omni Bead Ruptor 24 3D (2x 30s, liquid nitrogen in between). After adding an additional 1000 µL extraction mixture, the samples were shaken for 6 min at 4 °C and centrifuged at 20,000 g at 4 °C. New sterile reaction tubes containing the transferred 1ml of supernatant was stored at -80 °C. Metabolic profiling was performed in collaboration with the CEPLAS Plant Metabolism and Metabolomics Platform at Heinrich-Heine-Universität Düsseldorf using gas chromatography coupled to mass spectrometry.

3.5.4. Protein-based GC-MS Isotopologue Profiling

^{15}N feeding was performed with C_3 and C_4 *Cleome* plants as described in 3.2.2. Samples were freeze dried using a Beta 1-8 LDplus freeze dryer (Christ, Germany). The Protein-based GC-MS Isotopologue Profiling analysis performed in this study was adapted from a method described by Eylert et. al. (Eylert *et al.*, 2008). Plant material (~10 mg) was suspended in 500 µL of 6 M HCl. The samples were heated to 105 °C for ~24 h under an inert atmosphere before being briefly centrifuged. Hydrolysed samples were then dried under a stream of nitrogen at 70 °C. For additional purification dried samples were re-suspended in 200 µL acetic acid (98 %) and further purified via anion exchange solid phase extraction using Dowex 50Wx8. Polypropylene columns (1 mL Chromabond, Macherey-Nagel, Germany) were filled with 1 cm of washed Dowex and subsequently washed with 1 mL methanol and 1 mL MilliQ water. Samples were applied to the column, washed with 2 mL MilliQ water and eluted with 1 mL 4 M ammonium hydroxide. The eluents were dried at 70 °C under a stream of nitrogen. For measurement the residue was dissolved in 150 µL of 0.1 % Fac H_2O by vortexing for at least 12 s. Afterwards 15 µL of the sample was transferred into a glass inlet containing 50 µL MeOH and dried using a speed vac.

For derivatisation samples were resolved in a mixture of 50 µl dry acetonitrile and 50 µl N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (Sigma) and incubated at 70°C for 30 min. The resulting TBDMS derivatives were used for GC/MS analysis. GCMS parameter were modified after Shim *et al.* (Shim *et al.*, 2020). 1 µl of derivatized compounds was injected with an automatic liner exchange system in conjunction with a cold injection system (Gerstel) with a split ratio of 1:10 (ramping from 50 °C to 250 °C at 12 °C s-1) into the GC with a helium flow of 1 ml min-1. Chromatographic separation was performed using a 5977B GC/MSD system (Agilent Technologies) with a HP-5MS column with 5% phenyl methyl siloxane film (Agilent 19091S-433, 30 m length, 0.25 mm internal diameter, 0.25 µM film). The oven temperature was held constant at 70 °C for 2 min and then ramped at 12.5 °C min-1 to 320 °C at which it was held constant for 5 min; resulting in a total run time of 27 minutes. Metabolites were ionized with an electron impact source at 70 V and 200 °C source temperature and recorded in a mass range of m/z 60 to m/z 800 at 20 scans per second.

Owing to degradation by acid hydrolysis, the amino acids tryptophan, arginine and cysteine could not be analysed. Furthermore, acid hydrolysation led to the conversion of glutamine and asparagine to glutamate and aspartate. Therefore, results for aspartate and glutamate correspond to asparagine/aspartate and glutamine/glutamate, respectively.

Metabolites were identified via MassHunter Qualitative (v b08.00, Agilent Technologies) by comparison of spectra to the NIST14 Mass Spectral Library (https://www.nist.gov/srd/nist-standard-reference-database-1a-v14). A standard mixture containing all target compounds at a concentration

of 5 µM was processed in parallel to the samples as a response check and retention time reference. Peaks were integrated using MassHunter Quantitative (v b08.00, Agilent Technologies). Isotopologue data was corrected for natural isotope abundance using the Rtool Isocorrector (Heinrich *et al.*, 2018). Data analysis was performed according to (Antoniewicz, Kelleher and Stephanopoulos, 2007)Mol% for each metabolite were calculated based on the contribution of each individual isotopologue based on the analyzed fragments (s. Supplemental Figure 1).

3.5.5. Quantification of total N using Elemental Analysis – Isotope Ratio Mass Spectrometry (EA-IRMS)

Plant material was collected in 2 mL reaction tubes containing 3 glass beads and dried for 20 days at 60 °C. Dry samples were homogenized using an Omni Bead Ruptor 24 3D (Omni International, USA) and centrifuged at max. speed for 10 min to collect the plant material at the bottom of the tube. Using a XP6 Excellence Plus XP (6.1g x 0.1Ug) micro balance (Mettler Toledo, USA) 1.8 to 2.2 mg of plant material was weighed into small tin caps. The caps were folded, pressed, placed in a sealed 48-well-plate and stored until analysis on silica gel at 60 °C. Elemental analysis was performed in collaboration with the CEPLAS Plant Metabolism and Metabolomics Platform at Heinrich-Heine-Universität Düsseldorf using an IsoPrime100 isotope ratio mass spectrometry system.

3.5.6. Quantification of ^{15}N uptake using Isotope Ratio Mass Spectrometry (GC-IRMS)

^{15}N feeding was performed with C_3 and C_4 *Cleome* plants as described in 3.2.1. Samples for measuring uptake of ^{15}N were taken 4 h after transfer to either nutrient solution. Plant tissue was collected in 2 mL reaction tubes containing 3 glass beads. Sample preparation was performed as described for Elemental analysis of total N (see 3.6.6.), except for weighing 2.8 to 3.2 mg of plant material into each tin cap. Measurement of total N and ^{15}N content was performed at the University of Bonn in the lab of Prof. Dr. Gabriel Schaaf using GC-IRMS.

3.6. Expression Analysis

3.6.1. Phylogenetic analysis of nitrate reductase genes

The OrthoDB database was used to identify orthologs of *AtNIA1* and *AtNIA2* from various species. Coding sequences of orthologs from *Brassica napus, Brassica rapa, Zea mays* and *C. hassleriana* were downloaded from the NCBI and CoGe databases, respectively. Sequences of *C. gynandra* orthologs

were obtained using the CoGe BLAST too. All sequences were aligned using MAFFT alignment online tool. Phylogenetic trees were build using RAxMLGui tool based on MaximumLikelihood method (100 bs).

3.6.2. RNA extraction

Approximately 50 mg of frozen plant material was homogenized in 500 µL extraction buffer (80 mM Tris pH 9.0, 5 % SDS, 150 mM LiCl, 50 mM EDTA) and extracted with 500 µL of a phenol-chloroform-isoamyl alcohol-mix. After centrifugation at 14,000 g at RT for 25 min the upper aqueous phase was transferred to a new reaction tube. The extraction was subsequently repeated two more times. After the third extraction RNA was precipitated using 130 µL 8 M $LiCl_2$ and incubated overnight at -20 °C. The next day the RNA was pelleted by centrifugation at 14,000 g and 4 °C for 20 min. The pellet was dissolved in 300 µL H_2O by shaking at 65 °C for 10 min. After adding 100 µL $LiCl_2$ the samples were mixed and incubated overnight at -20 °C. The RNA was centrifuged at 14,000 g at 4 °C for 20 min. The pellet was washed in 400 µL ethanol (70 %) before being centrifuged at max. speed for 5 min. Finally, the supernatant was removed, and the pellet was dried and resolved in 30 µL water at 65°C for 20 min. RNA samples were stored at −20°C.

3.6.3. cDNA synthesis (Reverse Transcription)

The concentration of nucleic acids was measured spectrophotometrically, using a NanoPhotometer N60 (Implen, Germany). RNA concentration was adjusted to 800 ng/µl. 4 µl RNA was mixed with 0.5 µl DNAse buffer and 0.5 µl DNAse and incubated for 7 min at 37 °C. DNAse was inactivated via incubation at 70 °C for 5 min and subsequently stored on ice. cDNA was synthesized from the RNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher, USA) according to the manufacturer's instructions.

3.6.4. Real-time quantitative PCR (RT-qPCR)

The RT-qPCR was performed using a CFX96 Touch Real-Time PCR Detection System (BioRad, USA). Transcript levels were calculated relative to ACTIN transcript levels using the ΔCT method (Pfaffl, 2012). Sequences of primers used for the amplification of various genes are listed in Table 4.

Table 4: Oligonucleotides used in this study

Arabidopsis Locus	Name	Sequence	Name	Sequence
AT2G37620	ACT1_F2	ACTTCCCCATGCCATCCTAC	ACT1_R2	GCTCGTAGTCAAGGGCAATG
AT1G12110	NFP6.3_F	GTCGTCAACTGGGCATGTAC	NFP6.3_R	TGGACAAAGCAGCCATCAAG
AT2G38290	AMT2.1_F2	GGAGTCACCGGAAGAGAACA	AMT2.1_R2	GCAAAAGTGGAGACGAAGCA
AT1G37130	NIA2_1_F	GAGACCCACAACAAGAACGC	NIA2_1_R	TCCCTGTGAGACGGACCATC
	NIA2_2_F	GCACTACGTCCGCAACCAC	NIA2_2_R	GCTCCCCAGTTGAAGCCCT

4. Results

4.1. Nitrogen deficiency response varies between closely related *Brassicales* species with different photosynthesis types

4.1.1. Photosynthetic efficiency and biomass of C_4 *Cleome* plants is less affected by N deficiency

To investigate the effect of nitrogen deficiency in the context of C_4 photosynthesis, C_3 and C_4 *Cleome* plants were grown in controlled nutrient conditions using a vermiculite-based hydroponic system (s. 2.1). Plants were bottom watered with Hoagland medium with different nitrate concentrations (4 mM, 1 mM, 0.5 mM, 0.25 mM, 0.125 mM*). After 2 weeks the effect of N deficiency on the photosynthetic efficiency was determined by measuring CO_2 assimilation rates using an infrared gas exchange analyser (IRGA) (Figure 5).

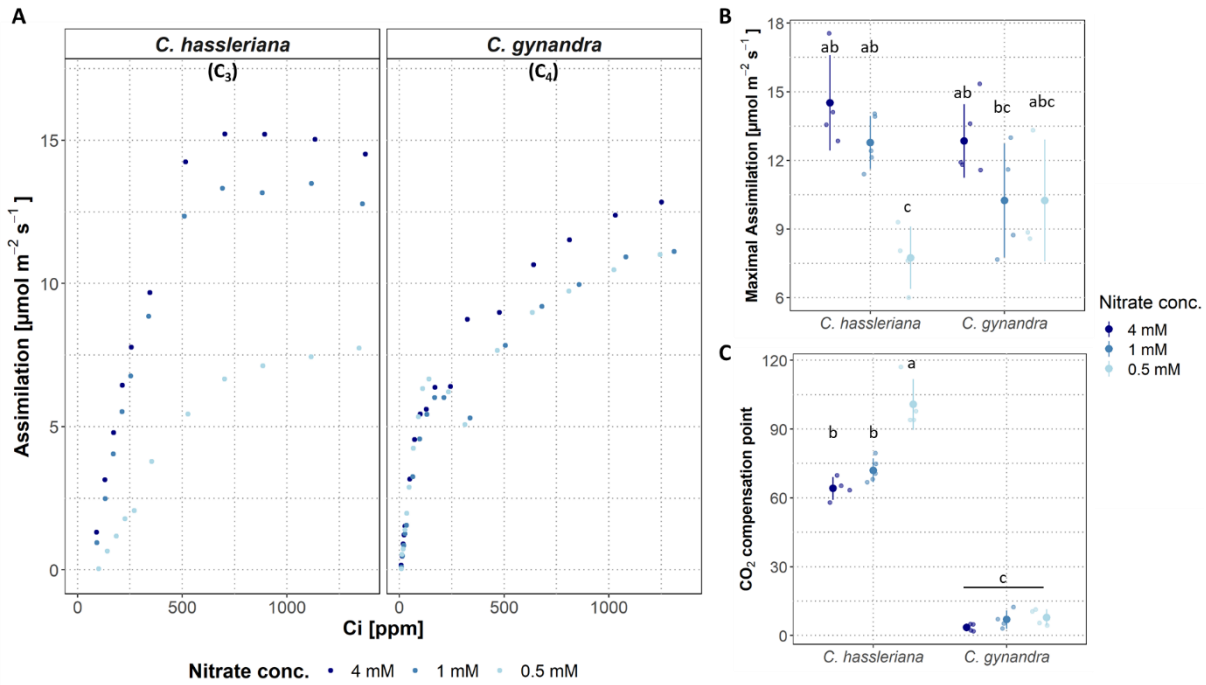

Figure 5: Comparative gas exchange measurements revealed a significant reduction of the photosynthesis rate in C_3 but not in C_4 species in response to N deficiency. A-Ci curves (A), maximal assimilation (B) and CO_2 compensation points (C) of *C. hassleriana* (C_3) and *C. gynandra* (C_4) plants grown on Hoagland solution with different nitrate concentrations (4 mM, 1 mM, 0.5 mM) (n=5).

Carbon assimilation was reduced in *C. hassleriana* grown in 0.5 mM nitrate medium compared to control conditions (4mM NO_3^-), while no change was observed in the C_4 species *C. gynandra* (Figure 5A). Moreover, the maximal assimilation and CO_2 compensation points were significantly reduced and increased, respectively, under N deficiency in the C_3 species while these parameters were not affected in the C_4 species (Figure 5B+C). Taken together these findings suggest that the photosynthetic efficiency of the C_4 species is less affected by N deficiency.

To investigate the effect of nitrogen deficiency on the biomass of the C_3 and C_4 *Cleome* species, the fresh weight of roots and shoots of plants grown in medium containing 4 mM, 1 mM, 0.5 mM and 0.25 mM nitrate was measured after 2 weeks of treatments. The total fresh weight was significantly reduced in all low nitrate conditions (1 mM, 0.5 mM, 0.25 mM) in the C_3 species. In contrast, the biomass of the C_4 species was not statistically reduced in response to the nitrate availability (Figure 6A).

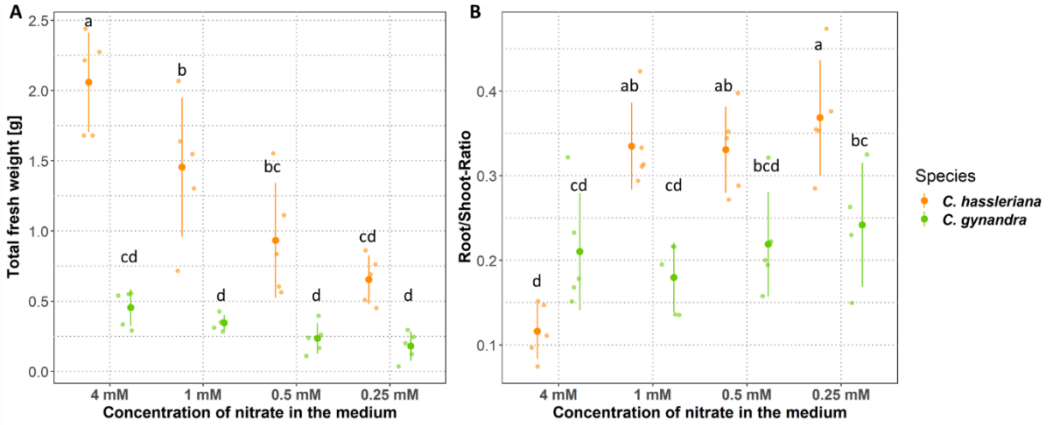

Figure 6: Biomass of C_4 *Cleome* species is less affected by N deficiency than close C_3 relative. Biomass (A) and Root/Shoot-Ratio (B) of *C. hassleriana* (C_3) and *C. gynandra* (C_4) plants grown on Hoagland solution with different nitrate concentrations (4 mM, 1 mM, 0.5 mM, 0.25 mM) (n=5).

The Root/Shoot-Ratio of *C. hassleriana* (C_3) showed a significant increase with decreasing nitrate concentration in the medium. In the C_4 species, the Root/Shoot-Ratio also increased, however, this increase was only significant in plants treated with the lowest nitrate concentration (Figure 6B).

C. gynandra is the most closely related C_4 species to the model organism *A. thaliana.* While the *Brassicaceae* family, which Arabidopsis is part of, does not contain any C_4 species, it includes multiple genera in which C_3C_4-intermediate photosynthesis evolved independently. C_3C_4-intermediate species represent a first step towards C_4 photosynthesis and therefore offer a great opportunity to study and characterize the early steps in the evolution of C_4 photosynthesis. For this reason, one C_3 species (*D. viminea, M. moricandioides*) and two C_3C_4-intermediate species (*D. muralis, D. tenuifolia, M. suffruticosa, M. arvensis*) from the *Diplotaxis* and *Moricandia* genera, respectively, were also included in this study. Similar to the *Cleome* species changes in biomass in response to N deficiency were analysed after two weeks (Figure 7).

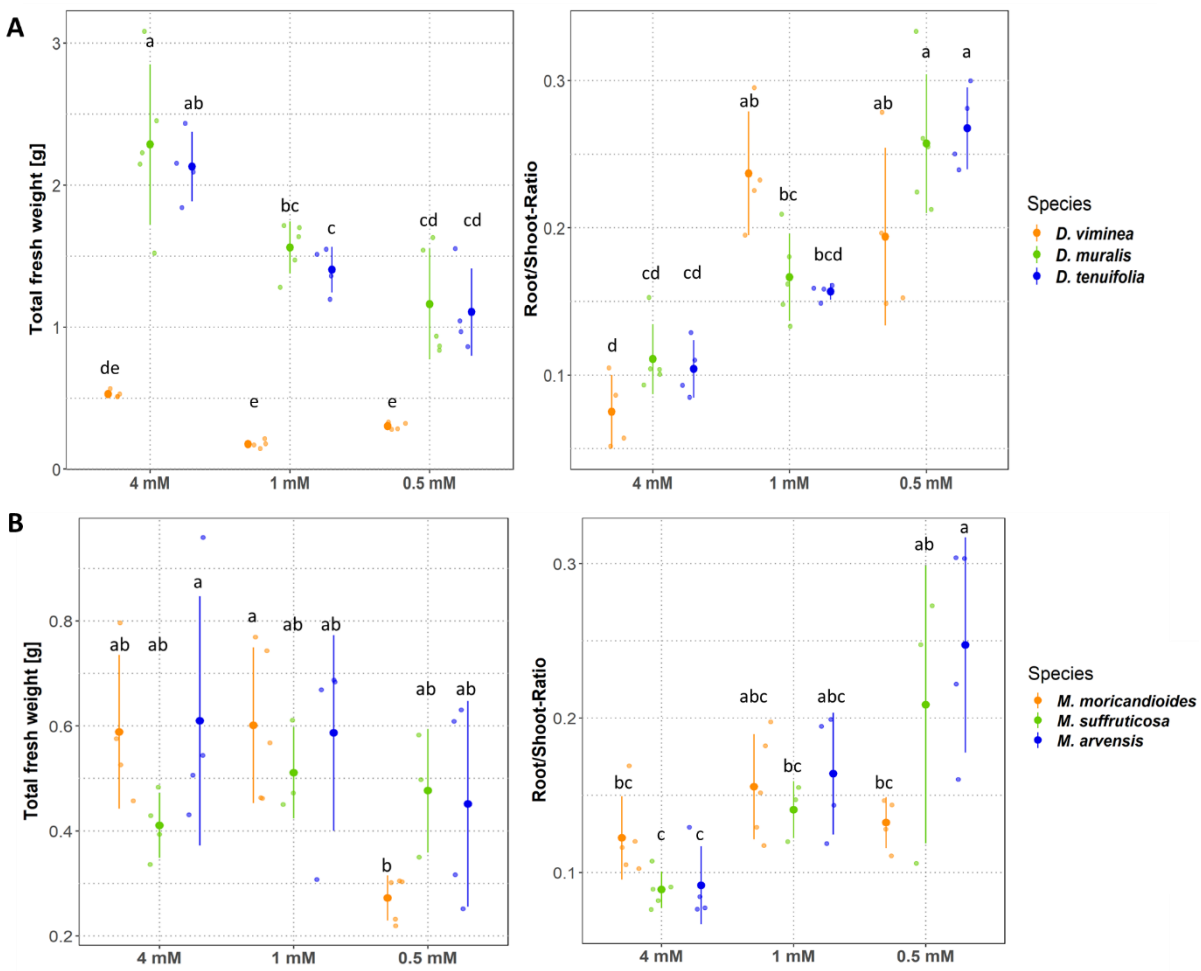

Figure 7: Fresh weight and Root/Shoot-Ratio of (A) *D. viminea* (C_3), *D. muralis* (C_3C_4) and *D. tenuifolia* (C_3C_4) and (B) *M. moricandioides* (C_3), *M. suffruticosa* (C_3C_4) and *M. arvensis* (C_3C_4) under full and low nitrogen conditions. Plants were bottom watered with Hoagland solution with different nitrate concentrations (4 mM, 1 mM, 0.5 mM). Biomass was measured after 2 weeks. (n=5)

The total fresh weight of all three *Diplotaxis* species was reduced in the lowest nitrate condition (0.5 mM), whereas among the *Moricandia* species, this was the case only for the C_3 species (Figure 7A+B). In the *Diplotaxis* genus, the Root/Shoot-Ratio increased in all species under low nitrate conditions (Figure 7A). However, in the two C_3C_4-intermediate species, this increase was more gradual than in the C_3 species and only significant in response to the lowest nitrate concentration (Figure 7A). In contrast, of the *Moricandia* species, only the two intermediates showed a significant increase in their Root/Shoot-Ratio in response to N deficiency.

Due to these surprising differences in biomass changes under N deficiency conditions between the *Cleome* and *Moricandia* C_3 species, CO_2 assimilation rates were also measured in the three *Moricandia* species. Since the *Moricandia* species were overall less sensitive to N deficiency, plants grown in 0.125 mM nitrate nutrient solution were used to investigate the effect on the photosynthetic efficiency.

Carbon assimilation was reduced in all three species grown in 0.125 mM nitrate nutrient solution compared to control conditions (Figure 8A).

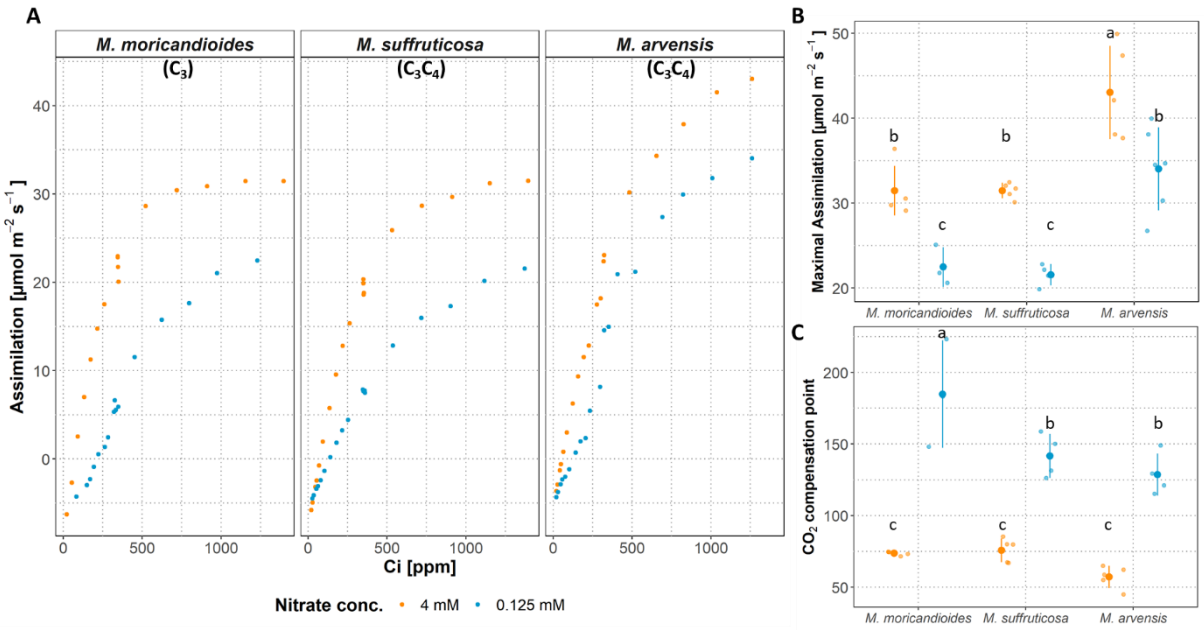

Figure 8: Comparative gas exchange measurements revealed differing CO_2 responses between species and treatments. A-Ci curves (A), Maximal Assimilation (B) and CO_2 compensation points (C) of *M. moricandioides* (C_3), *M. suffruticosa* (C_3C_4) and *M. arvensis* (C_3C_4) plants grown on Hoagland solution with high (4 mM) and low (0.125 mM) nitrate concentrations (n=5).

Consistent with this, the maximal assimilation was significantly lower under N deficiency than in control conditions (Figure 8B). Surprisingly, while the maximal assimilation of the C_3C_4-intermediate *M. arvensis* was also reduced in response to the reduced N availability, it was significantly higher than the maximal assimilation of both the other C_3C_4-intermediate species *M. suffruticosa* and the C_3 species *M. moricandioides* in both conditions (Figure 8B). The calculated CO_2 compensation points in control conditions showed no significant difference between the species (Figure 8). In response to the N deficiency treatment, the CO_2 compensation points increased significantly in all three species, with a stronger increase observed in the C_3 species compared to the two intermediates.

4.1.2. Quantification of anion content shows a higher allocation of NO_3^- from roots to shoots in C_4 species

To further investigate the underlying metabolic mechanisms causing these different growth patterns in response to N deficiency, anion content of roots and shoots was measured using IC. Anion contents in roots and shoots of individual species were then grouped by photosynthesis type and correlated to the different treatments (concentration of nitrate in the nutrient solution in which the plants were grown) (Figure 9). The correlation analysis revealed differences in the accumulation or distribution of nitrate, phosphate and sulphate in shoots and roots between all photosynthesis types. The C_3 species did not show any strong correlation besides the positive correlation between nitrate concentration in

the nutrient solution and nitrate content in both shoots and roots (Figure 9A+B). In contrast, in the intermediates, a strong negative correlation was observed between the nitrate concentration in the nutrient solution and phosphate and sulphate content in both roots and shoots. Finally, the correlations of anion content and nitrate concentration in nutrient solution differed between roots and shoots in the C_4 species.

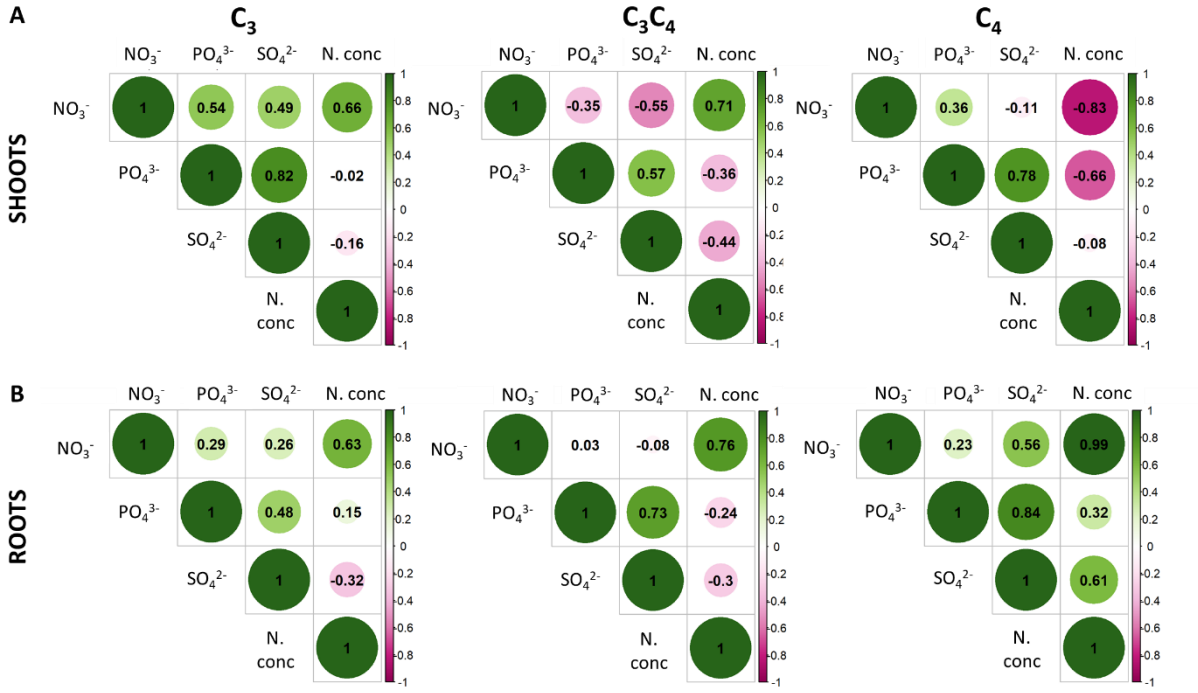

Figure 9: Correlation analysis of biomass and anion content of C_3, C_3C_4-intermediate and C_4 *Brassicales* species to nitrate deficiency. Correlation between NO_3^--concentration in the nutrient solution and shoot (A) and root (B) anion content grouped by photosynthesis type: C_3 (*C. hassleriana, D. viminea, M. moricandioides*), C_3C_4-intermediate (*D. muralis, D. tenuifolia, M. suffruticosa, M. arvensis*) and C_4 (*C. gynandra*) (n=5).

While root nitrate content showed a strong positive correlation to nitrate treatment, as observed in C_3 and intermediate species, the shoot nitrate content was negatively correlated to the nutrient solution nitrate concentration.

4.1.3. Distribution of total N content between roots and shoots differs in C_3 and C_4 *Cleome* species with higher relative N levels in shoots of the C_4 species

Next, we asked whether the C_4 cycle affects total N distribution between shoots and roots. EA IRMS analysis revealed that while the N contents in roots and shoots were similar in the C_3 species *C. hassleriana*, in the C_4 species significantly more N was found in the shoots (Figure 10A). The overall N content, however, was comparable in both species. N deficiency led to a decrease in total N in both species. Unlike in the C_3 species, which was affected already at the mild deficiency of 1 mM nitrate,

shoot N content in *C. gynandra* was significantly reduced only in response to the lowest nitrate concentration (0.25 mM). In contrast, the percentage of N in roots was already significantly reduced in the 1 mM condition. Overall, the C_3 species showed a more gradual decrease in total N content, that was equally distributed between both tissues.

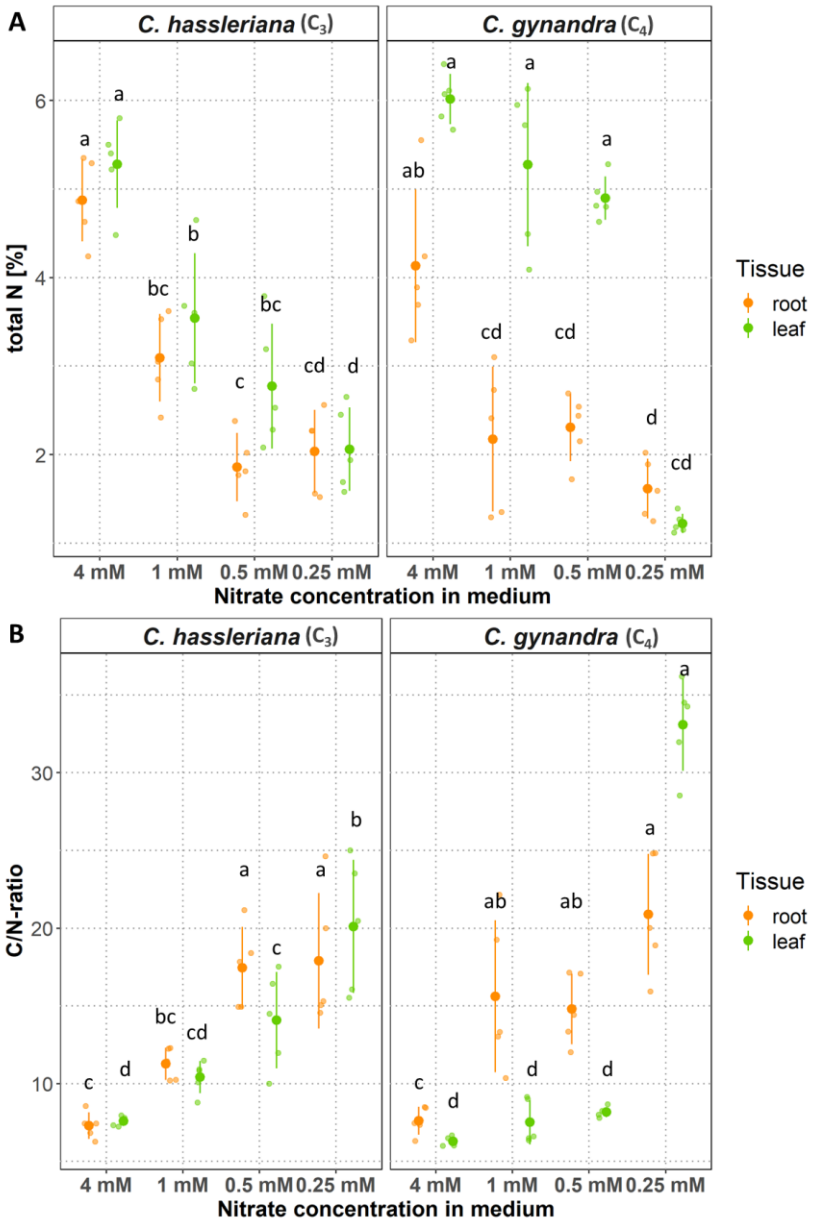

Figure 10: Total N content (A) and C/N-ratio (B) of roots and leaves of *C. hassleriana* (C_3) and *C. gynandra* (C_4) under full and low nitrogen conditions. Plants were bottom watered with Hoagland solution with different nitrate concentrations (4 mM, 1 mM, 0.5 mM, 0.25 mM). Total N content was measured after 2 weeks. (n=5)

C/N-ratios were calculated as an indicator of the nitrogen limitation of plants. Consistent with the decrease in total N content, the C/N-ratio in the C_3 species gradually increased with decreasing nitrate availability in the nutrient solution in both tissues, whereas in the C_4 species, the shoot C/N-ratio only increased significantly in the most severe N deficiency condition (Figure 10B).

4.1.4. Metabolic profiling reveals differential regulation of the TCA cycle between C_3 and C_4 *Cleome* species in both normal and low N condition

Previous analyses of biomass and anion content indicated an improved N deficiency tolerance of the C_4 species, possibly caused by a specific distribution of nutrients between roots and shoots. The observed negative correlation between the nitrate concentration in the nutrient solution and both NO_3^- and PO_4^{3-} accumulation points to an increase in their allocation to the shoots in response to low nitrate conditions. Consistently, EA IRMS analysis showed that in the C_4 species, significantly more N was found in the shoots while the overall N content, however, was comparable in both species. Unlike in the C_3 species, N content in *C. gynandra* was only significantly reduced in response to the lowest nitrate concentration (0.25 mM). Taken together, the increased allocation of N to the shoots in C_4 species indicates the presence of a more efficient root-to-shoot N transport mechanism compared to C_3 plants. To identify specific metabolites that show differences in either accumulation or allocation between the C_3 and C_4 species and to subsequently understand which pathways might be altered in their respective N deficiency responses, metabolic profiling using GCMS was performed with roots and shoots of C_3 and C_4 *Cleome* plants grown in normal and low N condition.

Metabolic profiling revealed clear differences between the species regarding the abundance of various groups of metabolites between roots and shoots in both normal and low N conditions (Figure 11).

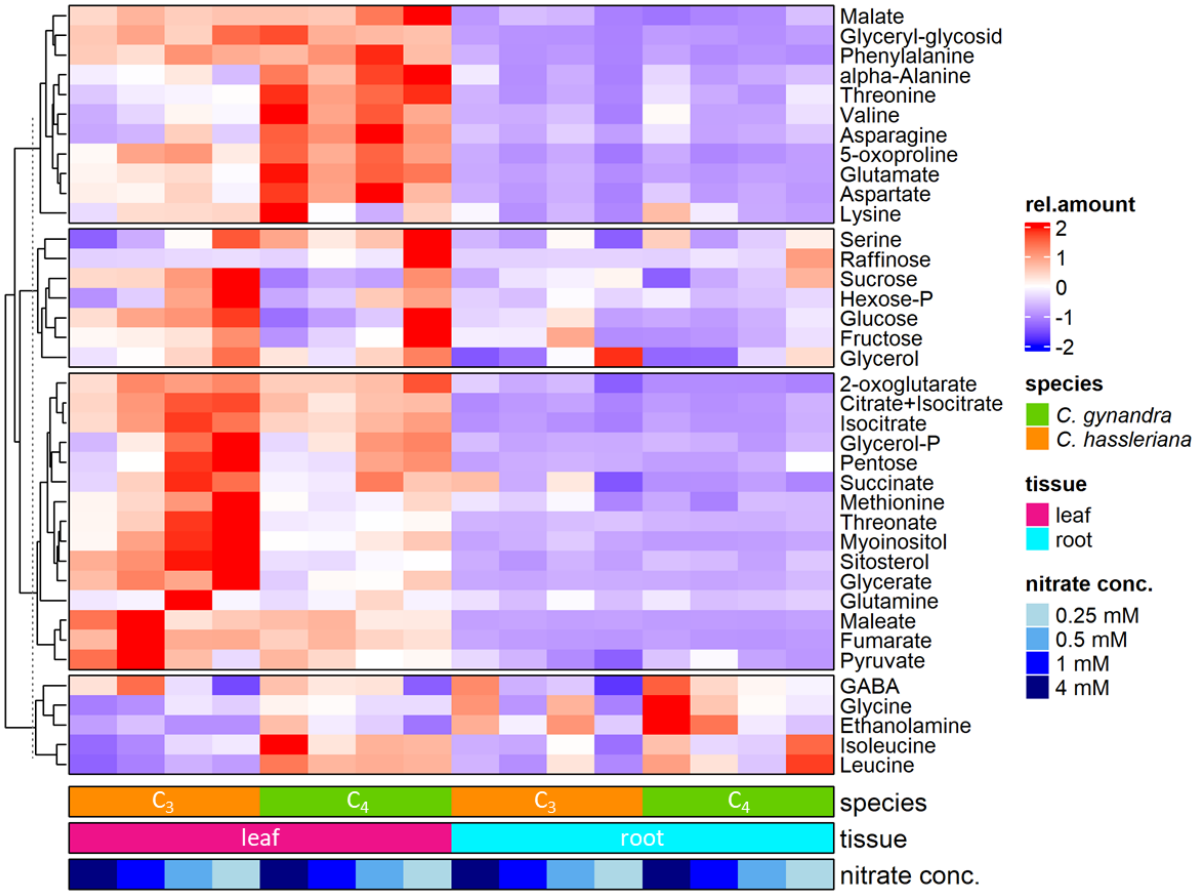

Figure 11: Heatmap representing the relative amounts of various metabolites in leaves and roots of *C. hassleriana* (C_3) and *C. gynandra* (C_4) under full and low nitrogen conditions. Relative amounts of metabolites were normalized using z-scores. Plants were bottom watered with Hoagland solution with different nitrate concentrations (4 mM, 1 mM, 0.5 mM, 0.25 mM). Metabolite profiling was performed after 2 weeks of treatment using GCMS. (n=4)

In both *Cleome* species, most metabolites were more abundant in leaves compared to roots. GABA, ethanolamine and the amino acids glycine, leucine, and isoleucine are an exception to this pattern as they accumulated in similar or in the case of the former three, in higher amounts in roots. In contrast, ethanolamine and glyceryl-glycoside were detected in significantly higher amounts in C_4 leaves. Most amino acids except glutamine, phenylalanine and methionine were more abundant in the leaves of the C_4 species in both control and N deficiency conditions.

As part of the metabolite profiling intermediates of the tricarboxylic acid (TCA) cycle were also quantified. Citrate and malate were the most abundant TCA cycle intermediates in the shoots and roots of both species. Unlike the sugars, the TCA cycle intermediates did not show a uniform pattern in response to N deficiency (Figure 12).

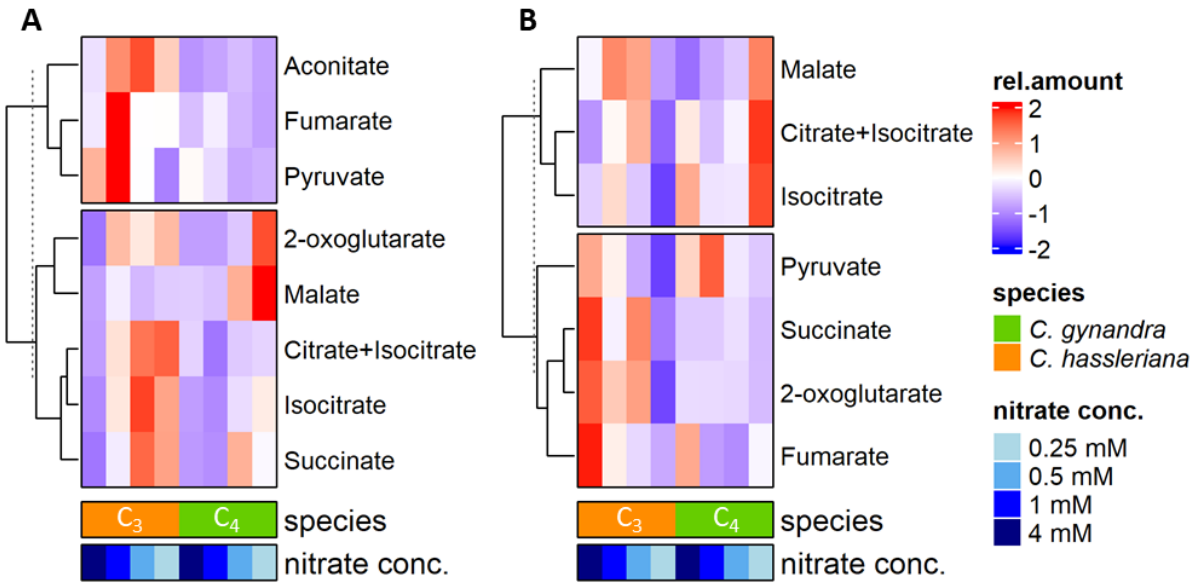

Figure 12: Heatmap representing the relative amounts of tricarboxylic acid cycle (TCA) intermediates in leaves of *C. hassleriana* (C_3) and *C. gynandra* (C_4) under full and low nitrogen conditions. Relative amounts of metabolites were normalized using z-scores. Plants were bottom watered with Hoagland medium with different nitrate concentrations (4 mM, 1 mM, 0.5 mM, 0.25 mM). Metabolite profiling was performed after 2 weeks of treatment. (n=4)

The analysis revealed a significant increase in relative levels of citrate, isocitrate and aconitate in shoots of the C_3 species during N deficiency. In contrast, in the C_4 species the amount of these three compounds did not change significantly (Figure 12A+B). Succinate and 2OG levels increased with decreasing N availability in both species. Malate content increased significantly with decreasing amounts of N in the medium in leaves of the C_4 species but not the C_3 species. Fumarate levels did not change in the C_4 species but showed a significant peak in 1 mM nitrate conditions in the C_3 species. C_3 leaves accumulated more pyruvate in 4 mM and 1 mM N conditions when compared to C_4 leaves in the same treatments. While the relative amount of pyruvate decreased in both species in low N conditions, the decrease was almost 10 times stronger in the C_3 species. Under control conditions, more pyruvate, fumarate, succinate and 2OG accumulated in the roots of C_3 plants compared to C_4 plants (Figure 12B). Levels of all TCA cycle intermediates were reduced in the strongest N deficiency treatment in C_3 but not C_4 roots. Only the amount of pyruvate and fumarate decreased in response to low N conditions while malate, citrate and isocitrate accumulated and levels of succinate and 2OG remained unchanged.

As part of the metabolic profiling, 15 of the 20 proteinogenic amino acids were measured using GCMS. Glutamate and aspartate were the most abundant amino acids in the roots and shoots of both species. Amino acid content in general was significantly higher in shoots compared to roots. Therefore, relative amounts of amino acids detected in shoots were normalized and presented in heatmap form separately (Figure 13).

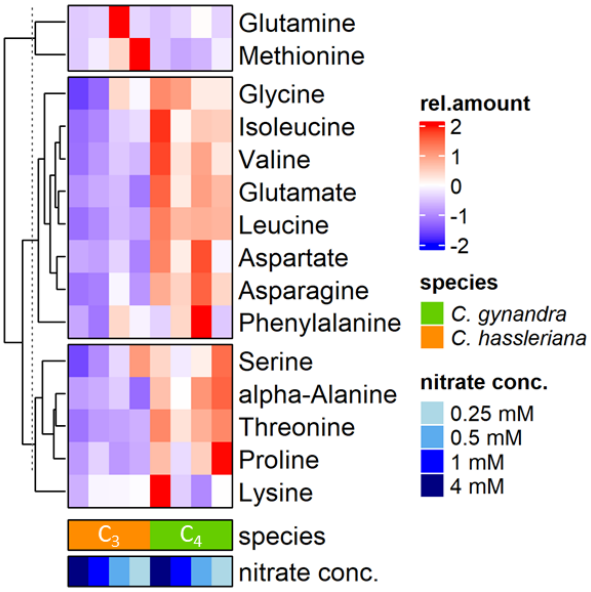

Figure 13: Heatmap representing the relative amounts of amino acids in leaves of *C. hassleriana* (C_3) and *C. gynandra* (C_4) under full and low conditions. Relative amounts of metabolites were normalized using z-scores. Plants were bottom watered with Hoagland medium with different nitrate concentration (4 mM, 1 mM, 0.5 mM, 0.25 mM). Metabolite profiling was performed after 2 weeks of treatment. (n=4)

Higher levels of leucine, threonine, glutamate, valine, isoleucine, asparagine, aspartate and alanine were found in leaves of the C_4 species when compared to C_3 leaves. Amongst these, leucine, valine and alanine are derived from pyruvate which was found to be reduced in both roots and shoots of *C. hassleriana* in response to N deficiency but only in roots in the C_4 species (not shown).

Under normal conditions, pyruvate levels are higher in C_3 plants (see above) but also show a stronger decrease than in C_4 plants under low N conditions. The pyruvate-derived amino acids show opposite trends in the C_3 and C_4 species in response to N deficiency. Relative amounts of leucine and valine decrease in the C_4 species but increase in the C_3 species with decreasing N availability (Figure 13). In contrast, alanine levels increased and decreased in low N conditions in C_4 and C_3 leaves, respectively. Also overall present in higher amounts in the C_4 species were aspartate and the aspartate-derived amino acids isoleucine, threonine and asparagine. Methionine and lysine however, illustrated different responses to low N conditions. Lysine, while also more abundant in control conditions in the C_4 species, decreased in C_4 leaves to levels similar to C_3 species in N deficiency conditions (0.25 mM). Even though, Methionine levels were similar in both species, they increased strongly in C_3 but not C_4 leaves under low N conditions. Alanine, phenylalanine, methionine, glutamine and glutamate were present in similar amounts in roots of C_3 and C_4 species under control conditions while all other amino acids were found in higher amounts in the C_4 species. Amounts of all detected amino acids were reduced in roots of C_3 plants in low N conditions, while in C_4 species, this was only the case for glycine, lysine, aspartate, alanine, phenylalanine, glutamate and glutamine. Glycine and serine, both derived from 3-phosphoglycerate (3-PGA) accumulated in the C_3 species under low N conditions but showed opposing

trends in the C_4 species with glycine decreasing and serine increasing in response to N deficiency (Figure 14A).

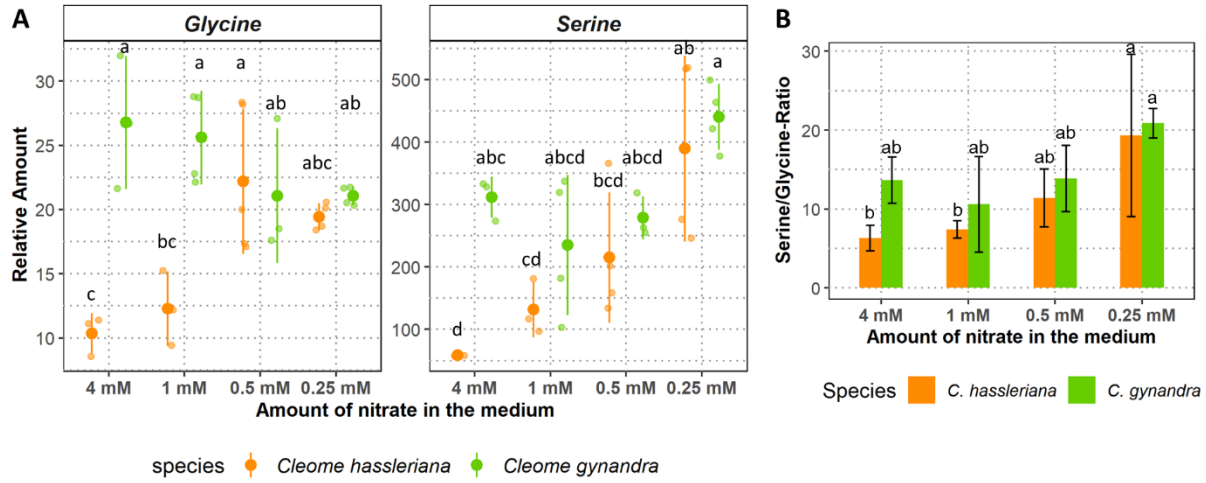

Figure 14: Metabolite profiling of *C. hassleriana* (C_3) and *C. gynandra* (C_4) shoots under decreasing nitrogen conditions reveals differences in glycine and serine levels in response to N deficiency. Graphs show the amount of (A) glycine and serine and (B) Serine/Glycine-Ratio in leaves of C_3 and C_4 *Cleome* species. Plants were bottom watered with Hoagland solution medium with different nitrate concentrations (4 mM, 1 mM, 0.5 mM, 0.25 mM). Metabolite profiling was performed after 2 weeks of treatment. (n=4)

Serine/Glycine-Ratio in leaves was calculated from serine and glycine content within each biological replicate. In control conditions (4 mM) the Serine/Glycine-Ratio is higher in C_4 species compared to C_3 species. With decreasing N availability ratios increased more strongly in shoots in the C_3 species than in the C_4 species (Figure 14B).

Metabolic profiling further revealed the accumulation of various sugars in both species mainly in the leaves during N deficiency (Figure 15).

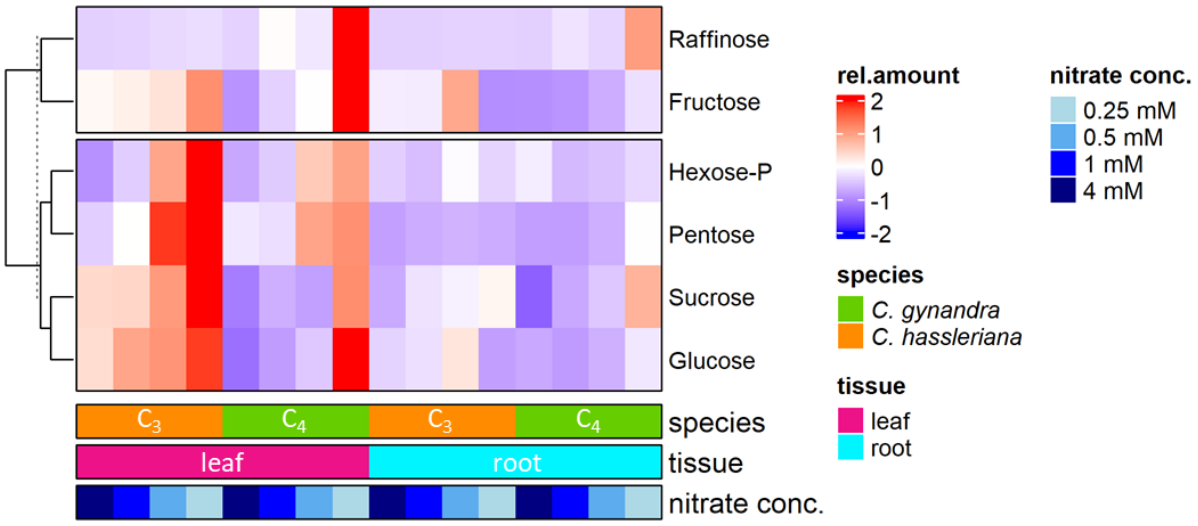

Figure 15: Heatmap representing the relative amounts of sugars in leaves of *C. hassleriana* (C_3) and *C. gynandra* (C_4) under full and low conditions. Relative amounts of metabolites were normalized using z-scores. Plants were bottom watered with Hoagland medium with different nitrate concentration (4 mM, 1 mM, 0.5 mM, 0.25 mM). Metabolite profiling was performed after 2 weeks of treatment. (n=4)

Except for raffinose, sugar levels were generally higher in the C_3 species when compared to the C_4 species (Figure 15). Raffinose was found in significantly lower amounts than the other sugars and only increased significantly in roots and shoots of *C. gynandra* in the 0.25 mM treatment (Figure 15). Pentose levels showed a significant increase with decreasing nitrate concentration in the medium in C_3 shoots and C_4 roots, respectively (Figure 15). The shoot pentose content also increased in the C_4 species in the two lowest N deficiency treatments, however, this change was not significant. Both glucose and fructose levels increased only significantly in the C_4 species. Levels of these two sugars in roots did not show any clear trends or significant changes in either species (Figure 15). Sucrose was the only sugar found to be significantly increased in shoots of both species and also showed an increase in roots in response to N deficiency (Figure 15).

Various other metabolites also showed different responses to N deficiency in C_3 and C_4 species (Figure 16).

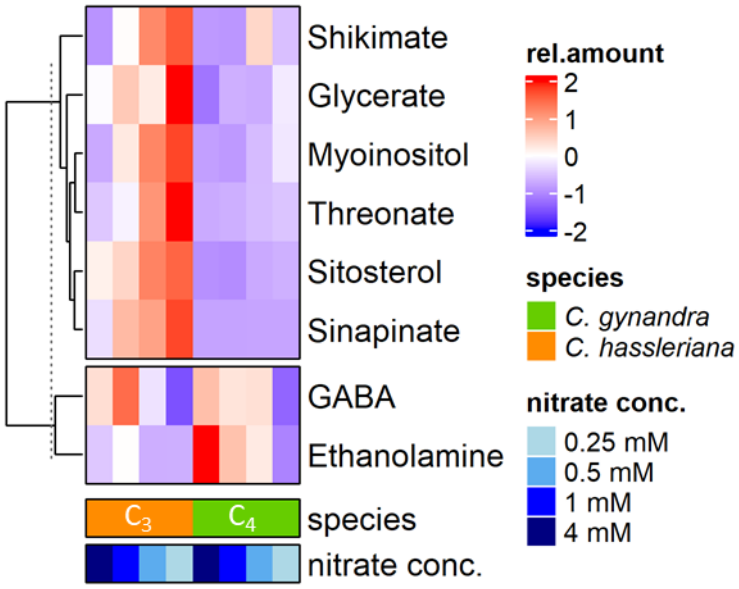

Figure 16: Heatmap representing the relative amounts of various metabolites in leaves of *C. hassleriana* (C_3) and *C. gynandra* (C_4) under full and low nitrogen conditions. Relative amounts of metabolites were normalized using z-scores. Plants were bottom watered with Hoagland medium with different nitrate concentrations (4 mM, 1 mM, 0.5 mM, 0.25 mM). Metabolite profiling was performed after 2 weeks of treatment. (n=4)

A group of metabolites including shikimate, glycerate, myoinositol, threonate, sitosterol and sinapinate accumulated significantly only in shoots of the C_3 species in low N conditions (Figure 16). Under low N conditions, GABA shoot content was reduced in both species. Ethanolamine levels, which were higher in the C_4 species under control conditions, decreased only in the C_4 leaves.

Overall, the metabolite profiling revealed differences in the amounts of a broad variety of metabolites including amino acids, sugars and TCA cycle intermediates under both normal and low N conditions. Furthermore, differences between the changes in metabolite levels between the control and N deficiency treatments observed between the C_3 and C_4 species could indicate differential regulation in response to N deficiency.

4.2. Ammonium tolerance/sensitivity varies between genera

Plants can take up inorganic N in the form of nitrate (NO_3^-) and ammonia (NH_4^+) (Miller and Cramer, 2005) but differ in the preference of these sources. Due to their higher rates of photorespiration, C_3 plants generally have a bigger NH_4^+ pool relative to C_4 plants. Regarding their N source preference, this could mean that they are better adapted to using NH_4^+ as an N source than their C_4 counterparts, since their metabolism might already be adapted to higher NH_4^+ levels. However, the increased pool of photorespiratory NH_4^+ could also limit the amount of additional NH_4^+ C_3 plants can withstand and

potentially make them more sensitive to NH_4^+. To test which of these two hypotheses is true C_3 and C_4 *Cleome* plants were grown in a gradient of NH_4^+ concentrations.

4.2.1. Biomass of C_3 and C_4 *Cleome* species is unaffected by increasing ammonium concentration

To investigate whether the ability to use different nitrogen sources varies between C_3 and C_4 species, C_3 and C_4 *Cleome* plants were grown in nutrient solutions containing both nitrate (NO_3^-) and ammonium (NH_4^+) in different compositions, including 0 %, 10 %, 25 %, 50 %, 90 % and 100 % ammonium. Biomass was measured after 2 weeks. No significant changes in biomass between the 0 % and 100 % NH_4^+ nutrient solutions were observed (Figure 17).

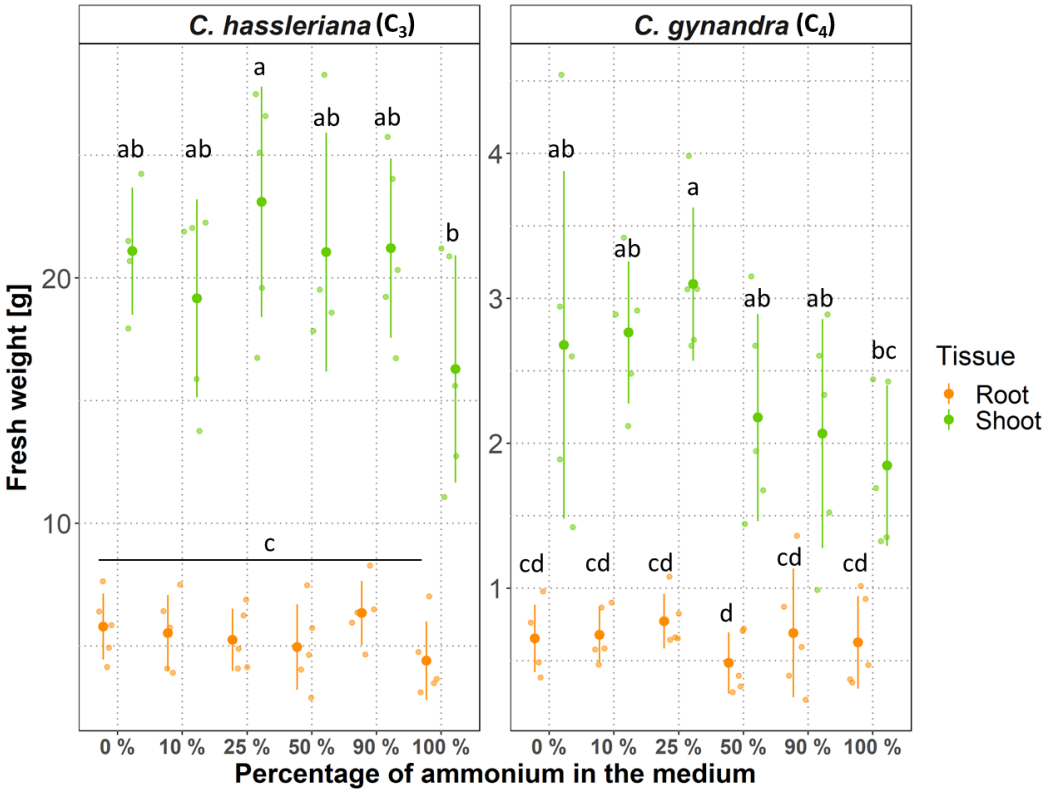

Figure 17: Biomass of *C. hassleriana* (C_3) and *C. gynandra* (C_4) grown in media with different compositions containing both nitrate and ammonium as nitrogen sources. Plants were bottom watered with Hoagland solution with different amounts of nitrate and ammonium, percentages indicate the portion of overall nitrogen in the nutrient solution made up of ammonium (0 %, 10 %, 25 %, 50 %, 90 %, 100 %). Biomass was measured after 2 weeks. (n=5)

Both species achieved the highest biomass when provided with a mixture of NO_3^- and NH_4^+ and the lowest when grown in the 100 % NH_4^+ solution (Figure 17). Both species grew best in the 25 % nutrient solutions, the strongest and only significant decrease was observed between the 25 and 100 %.

However, the decrease in biomass in the 50 % and 90 % treatments was stronger in the C_4 than the C_3 species, which could imply that *C. gynandra* might be more sensitive to high NH_4^+ concentrations.

4.2.2. Biomass of *Moricandia* and *Diplotaxis* species is reduced when grown with ammonium as their main N source

In C_3C_4-intermediate species, photorespiration is restricted to BS cells while photorespiratory NH_3 is recaptured via the photorespiratory nitrogen cycle in the mesophyll cells, which results in the necessity of metabolic rerouting to balance these changes and might allow for more efficient use or higher tolerance to additional NH_4^+ supplied in the nutrient solution. NH_4^+ supply experiments were also conducted using one C_3 and two C_3C_4 intermediate species from the *Moricandia* and *Diplotaxis* genera to address how NH_4^+ assimilation in C_3C_4 intermediates differs from that of C_3 plants. Biomass was measured after 2 weeks of treatment. Except for *D. tenuifolia,* which has the lowest CO_2 compensation point (CO_2CP) of all tested species, a decrease in total biomass with increasing NH_4^+ concentration was observed (Figure 18).

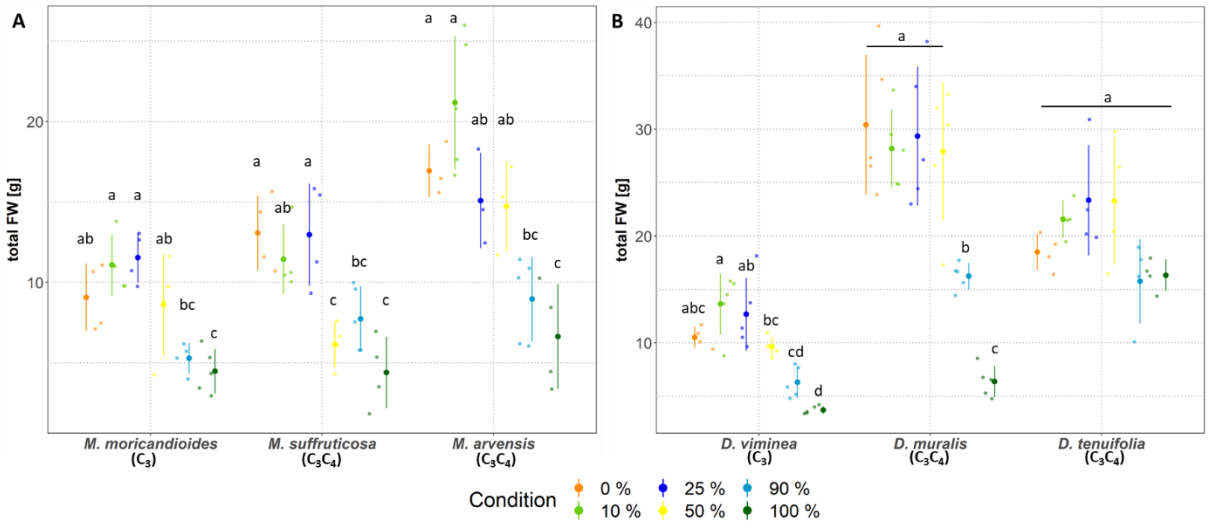

Figure 18: Biomass of (A) *M. moricandioides* (C_3), *M. suffruticosa* (C_3C_4), *M. arvensis* (C_3C_4) and (B) *D. viminea* (C_3), *D. muralis* (C_3C_4), *D. tenuifolia* (C_3C_4) grown in media with different compositions containing both nitrate and ammonium as nitrogen sources. Plants were bottom watered with Hoagland solution with different amounts of nitrate and ammonium, percentages indicate the portion of overall nitrogen in the medium made up of ammonium (0 %, 10 %, 25 %, 50 %, 90 %, 100 %). Biomass was measured after 2 weeks. (n=5)

While not significant, *D. tenuifolia* showed a slight increase in biomass in the 25 % and 50 % treatments and relatively lower fresh weights in the two treatments with the highest NH_4^+ concentration (Figure 18B). The two C_3 species *M. moricandioides* and *D. viminea* showed a slight increase in biomass in the 10 % and 25 % treatments compared to the standard Hoagland solution (0 %).

4.2.3. Anion content is significantly altered in the presence of ammonium

Anion content in roots and shoots of plants grown in the different nutrient solutions was analysed after 2 weeks using IC. Levels of NO_3^-, PO_4^{3-} and SO_4^{2-} in shoots and roots of C_3 and C_4 *Cleome* species changed significantly when grown in nutrient solutions containing both nitrate (NO_3^-) and ammonium (NH_4^+) in different compositions, including 0 %, 10 %, 25 %, 50 %, 90 % and 100 % NH_4^+ (Figure 19). The NO_3^- content significantly decreased in the two treatments with the highest NH_4^+ percentage (90 %, 100 %) in roots and shoots of both species (Figure 19A).

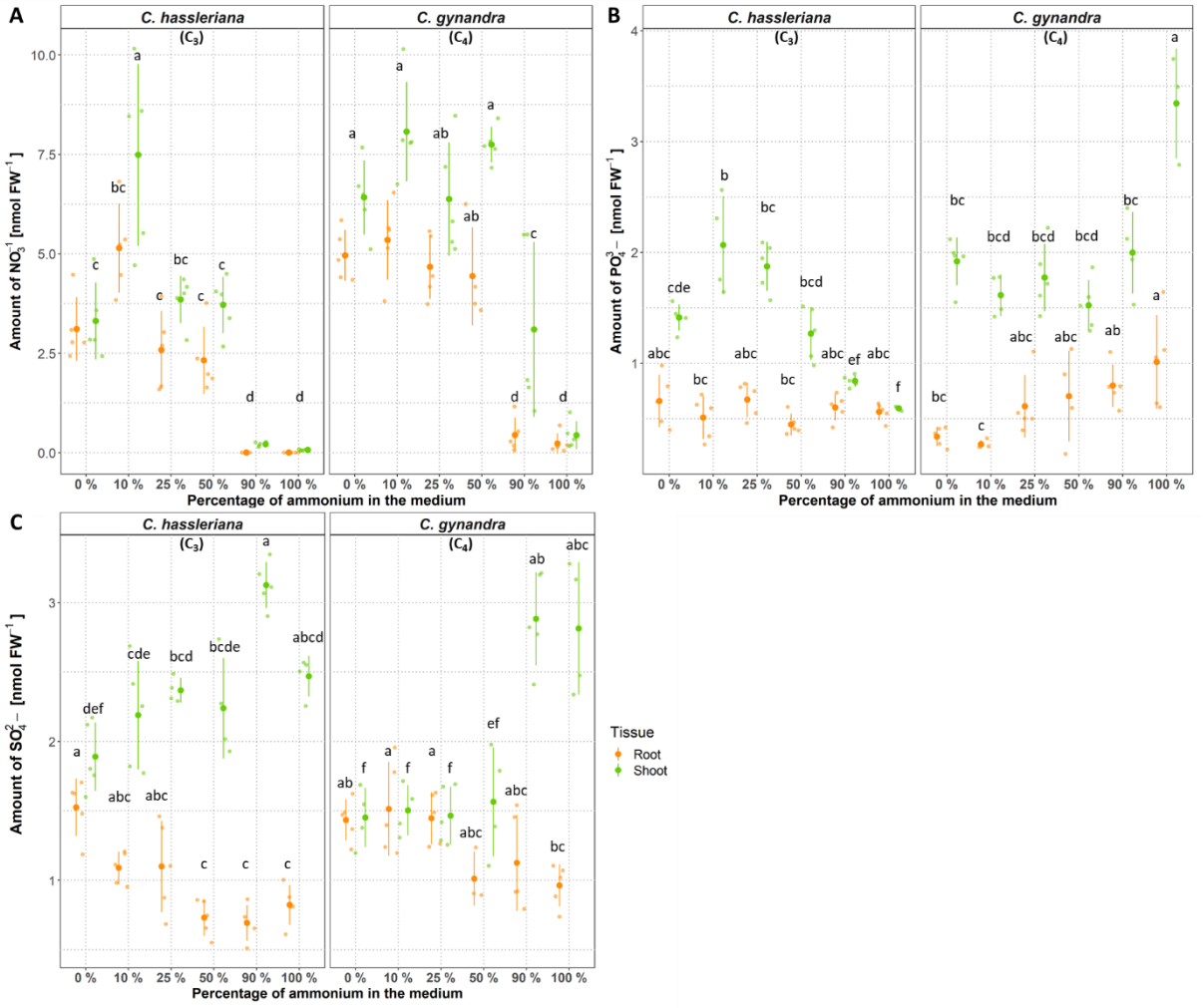

Figure 19: Anion content of *C. hassleriana* (C_3) and *C. gynandra* (C_4) grown in nutrient solutions with different compositions of nitrate and ammonium as nitrogen sources. Plants were bottom watered with Hoagland solution with different amounts of nitrate and ammonium, percentages indicate the portion of overall nitrogen in the medium made up of ammonium (0 %, 10 %, 25 %, 50 %, 90 %, 100 %). Nitrate (A), phosphate (B) and sulphate (C) content was measured after 2 weeks. (n=5)

While the C_4 species does not show any significant changes in NO_3^- levels between the first 4 treatments, significantly higher NO_3^- content was observed in the 10 % NH_4^+ treatment in the shoots and roots of *C. hassleriana* (C_3) (Figure 19A). In C_3 leaves, PO_4^{3-} content, similar to the NO_3^-content, was highest in the 10 % NH_4^+ treatment and decreased with increasing NH_4^+ concentration of the nutrient

solution, while the PO_4^{3-} content of the roots did not change (Figure 19B). In contrast, C_4 roots showed a significant gradual increase in root PO_4^{3-} content with the increasing amount of NH_4^+ in the nutrient solution. The leaf PO_4^{3-} content of the C_4 species was significantly increased only in 100 % NH_4^+ solution. Leaf SO_4^{2-} content increased with increasing NH_4^+ percentage in both species (Figure 19C). However, in *C. hassleriana* SO_4^{2-} content in the 100 % NH_4^+ treatment was reduced compared to the 90 % treatment.

To better understand how the underlying mechanisms might differ between the C_3 and C_3C_4-intermediate species, the anion content in roots and shoots of the *Moricandia* and *Diplotaxis* species was also measured. Plants grown in the different nutrient solutions were analysed after 2 weeks using IC. NO_3^- content and its distribution differed between species and treatments, but overall decreased with increasing NH_4^+ concentration, i.e. decreasing NO_3^- concentration in the nutrient solution as expected (Figure 20).

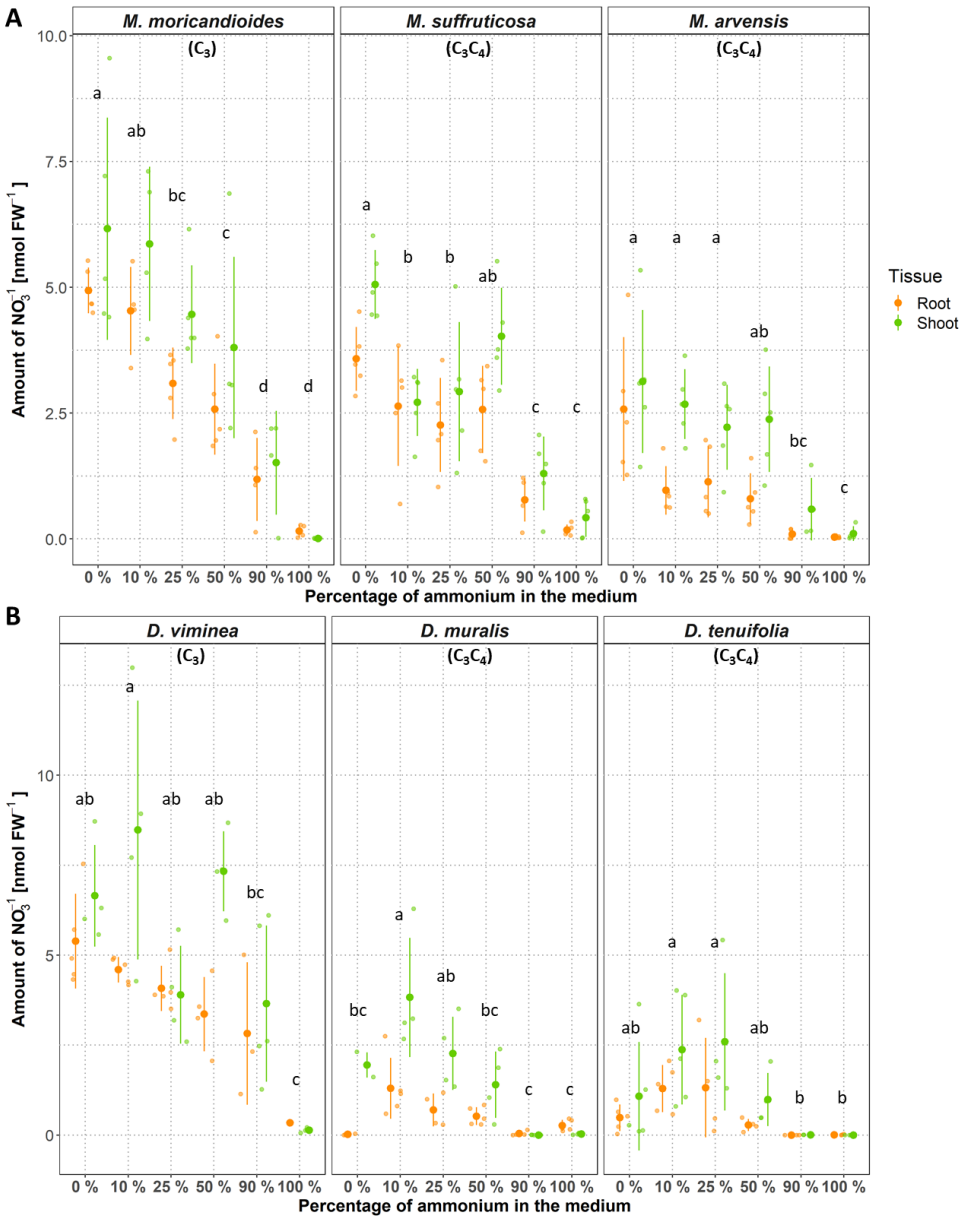

Figure 20: Nitrate content of (A) *M. moricandioides* (C_3), *M. suffruticosa* (C_3C_4), *M. arvensis* (C_3C_4) and (B) *D. viminea* (C_3), *D. muralis* (C_3C_4), *D. tenuifolia* (C_3C_4) grown in media with different compositions containing both nitrate and ammonium as nitrogen sources. Plants were bottom watered with Hoagland media with different amounts of nitrate and ammonium, percentages indicate the portion of overall nitrogen in the medium made up by ammonium (0 %, 10 %, 25 %, 50 %, 90 %, 100 %). Nitrate content was measured after 2 weeks using IC. (n=5)

In contrast to the *Moricandia* species, which all showed their highest NO_3^- accumulation in the full NO_3^- treatment (0 %) (Figure 20A), the NO_3^- concentration was highest in the 10 % NH_4^+ treatment in all three *Diplotaxis* species (Figure 20B). In both genera, the decrease in NO_3^- levels between the 50 % and 90 % treatment was smaller in the two C_3 species than the C_3C_4-intermediates.

Unlike in the two *Cleome* species no clear trends were observed for PO_4^{3-} and SO_4^{2-} levels (not shown). However, the leaf chloride (Cl^-) levels showed a strong increase in response to higher NH_4^+

concentrations in the nutrient solution (Figure 21A). Root chloride content also increased, albeit not significantly in all species.

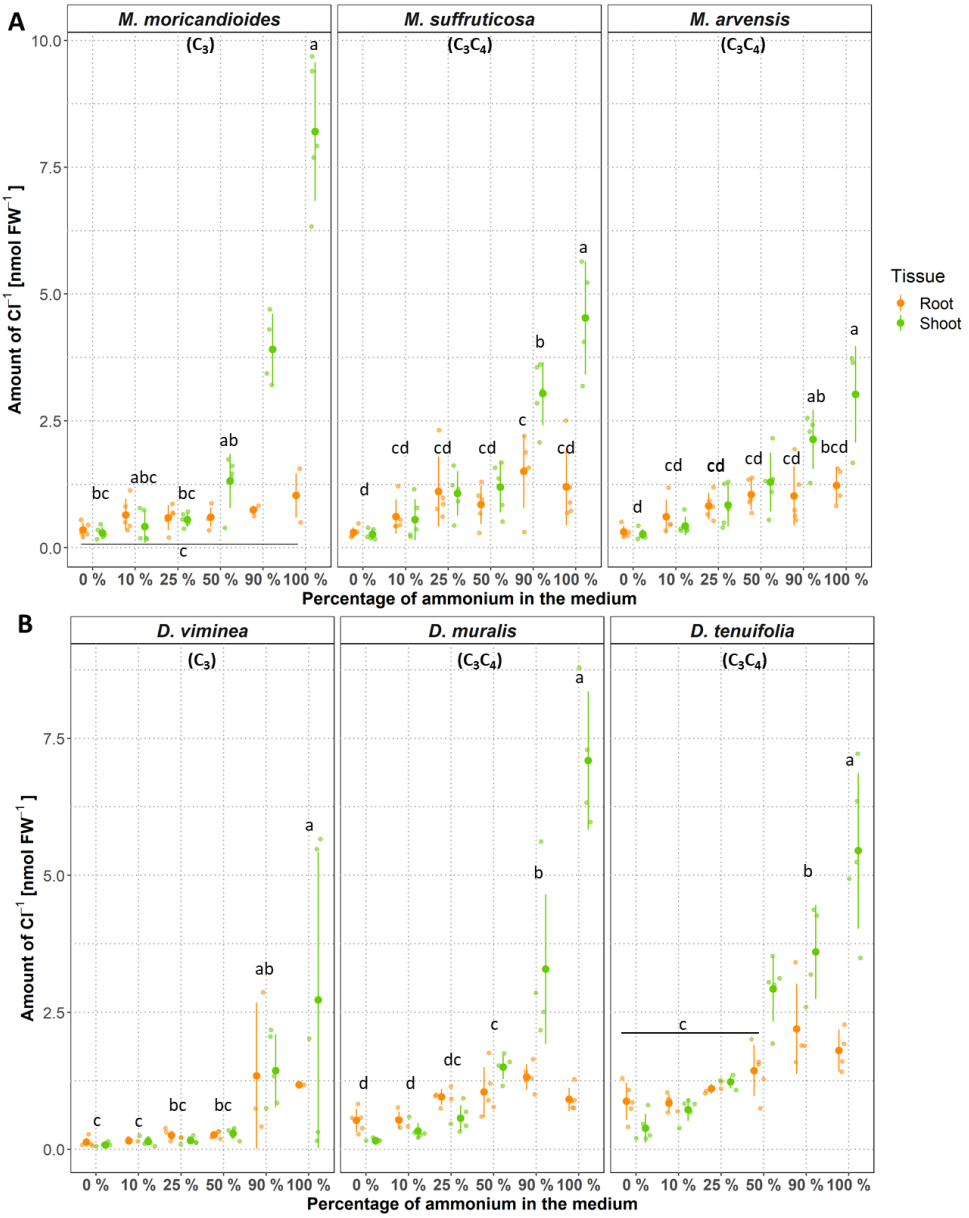

Figure 21: **Chloride content of (A) *M. moricandioides* (C_3), *M. suffruticosa* (C_3C_4), *M. arvensis* (C_3C_4) and (B) *D. viminea* (C_3), *D. muralis* (C_3C_4), *D. tenuifolia* (C_3C_4) grown in media with different compositions containing both nitrate and ammonium as nitrogen sources.** Plants were bottom watered with Hoagland media with different amounts of nitrate and ammonium, percentages indicate the portion of overall nitrogen in the medium made up by ammonium (0 %, 10 %, 25 %, 50 %, 90 %, 100 %). Chloride content was measured after 2 weeks using IC. (n=5)

As shown in the N deficiency experiment (4.1.2, Figure 9), shoot and root anion content (NO_3^-, SO_4^{2-}, PO_4^{3-}) of individual species was then grouped by photosynthesis type and correlated to the different treatments (concentration of nitrate in the nutrient solution in which the plants were grown). The nitrate concentration was inversely proportional to the amount of ammonium. Therefore, the effect of ammonium can be further investigated by comparing the correlation to the results from the N deficiency experiment (Figure 22).

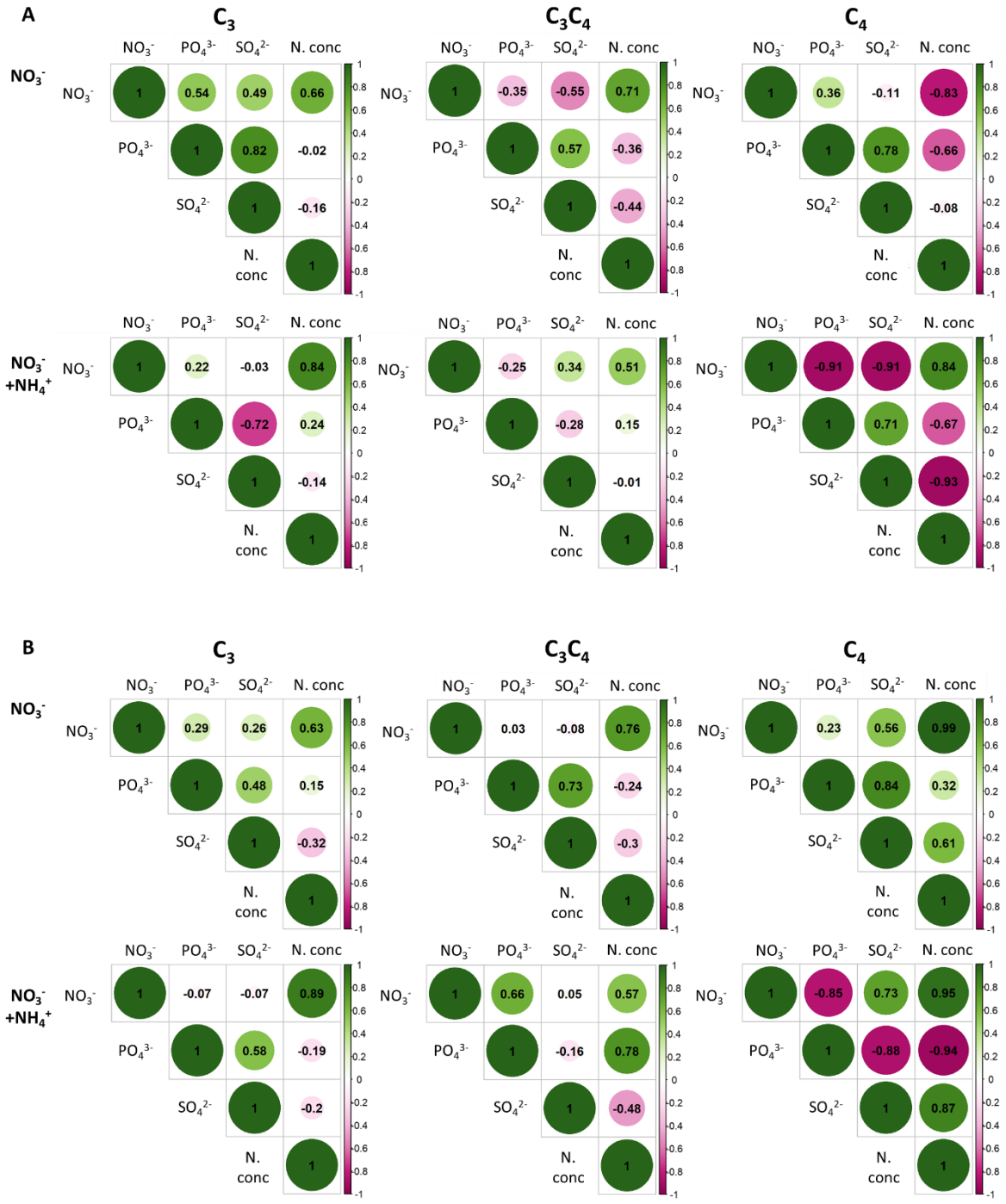

Figure 22: Correlation analysis of anion content of C_3, C_3C_4-intermediate and C_4 *Brassicaeceae* species to nitrate concentration in growth medium with and without ammonium. Correlation between NO_3^--concentration in the medium and shoot (A) and root (B) anion content grouped by photosynthesis type: C_3 (*C. hassleriana, D. viminea, M. moricandioides*), C_3C_4-intermediate (*D. muralis, D. tenuifolia, M. suffruticosa, M. arvensis*) and C_4 (*C. gynandra*) (n=5).

Compared to the experiment without NH_4^+, a stronger positive correlation between NO_3^- concentration of the nutrient solution and NO_3^- content in shoots was observed in C_3 plants grown in NH_4^+-containing nutrient solution. The correlation analysis further revealed a strong negative correlation between PO_4^{3-} and SO_4^{2-} levels in shoots, only present in plants grown in NH_4^+ medium (Figure 22A). As seen in shoots,

the positive correlation between the available NO_3^- and root NO_3^- content was increased in the C_3 species compared to the plants grown on medium without NH_4^+. Neither PO_4^{3-} nor SO_4^{2-} content was strongly correlated to NO_3^- concentration as was the case in the absence of NH_4^+ (Figure 22B).

In C_3C_4 intermediate species nitrate content in both roots and shoots showed a weaker positive correlation to the NO_3^- concentration in the medium (Figure 22A+B). The presence of NH_4^+ seems to increase and decrease SO_4^{2-} and PO_4^{3-} uptake, respectively (Figure 22B).

In C_4 species, the strong negative correlation of NO_3^- content in shoots to the NO_3^- media concentration observed in NO_3^- only medium is inverted when NH_4^+ was added to the medium (Figure 22A). Furthermore, PO_4^{3-} and SO_4^{2-} content showed a strong negative correlation to the nitrate concentration in the nutrient solution, which was only observed for PO_4^{3-} in the previous experiment. Root NO_3^- levels showed a strong positive correlation to the available NO_3^- in both experiments. PO_4^{3-} and SO_4^{2-} content in roots was strongly negatively and positively correlated to the NO_3^-, respectively (Figure 22B). Similarly, in analysis of the shoots, this was only the case for SO_4^{2-} in the previous experiment without NH_4^+. Taken together this implies that NH_4^+ not only affects NO_3^- uptake and assimilation but also the assimilation of PO_4^{3-} and SO_4^{2-} under nitrate starvation.

4.2.4. C_4 *Cleome* species profit less from ammonium supplementation than their C_3 relatives

The previous experiments showed that nutrient solution containing 25 % NH_4^+ improved the growth of both *Cleome* species relative to growth with NO_3^- as their sole N source. However, the species responses to higher NH_4^+ concentrations differed between the species indicating differences in NH_4^+ sensitivity. Changes in the correlation of the anion content compared to the NO_3^--only experiment point to these changes being related to differences in the assimilation of NH_4^+. Taken together these findings could indicate a lower NH_4^+ tolerance of C_4 plants due to changes in N metabolism resulting from C_4 photosynthetic evolution e.g. a smaller pool of photorespiratory NH_4^+. The interplay between NH_4^+ sensitivity and N deficiency tolerance was further investigated by combining these factors in one experiment. Plants from 6 treatments were analysed: (1) nitrate only (4 mM N), (2) ammonium only (4 mM A), two N deficiency treatments ((3) 1 mM N; (5) 0.5 mM N) and two treatments that contained the same amount of nitrate as the deficiency treatments but were supplemented with ammonium to reach a combined concentration of 4 mM ((4) 1 mM N+ 3 mM A); (6) 0.5 mM N+ 3.5 mM A).

To test whether changes observed in anion content in the presence of NH_4^+ are directly linked to photosynthesis type, CO_2 assimilation rates of C_3 and C_4 *Cleome* plants grown in different compositions of NH_4^+ and NO_3^- were measured after 2 weeks using IRGA (Figure 23).

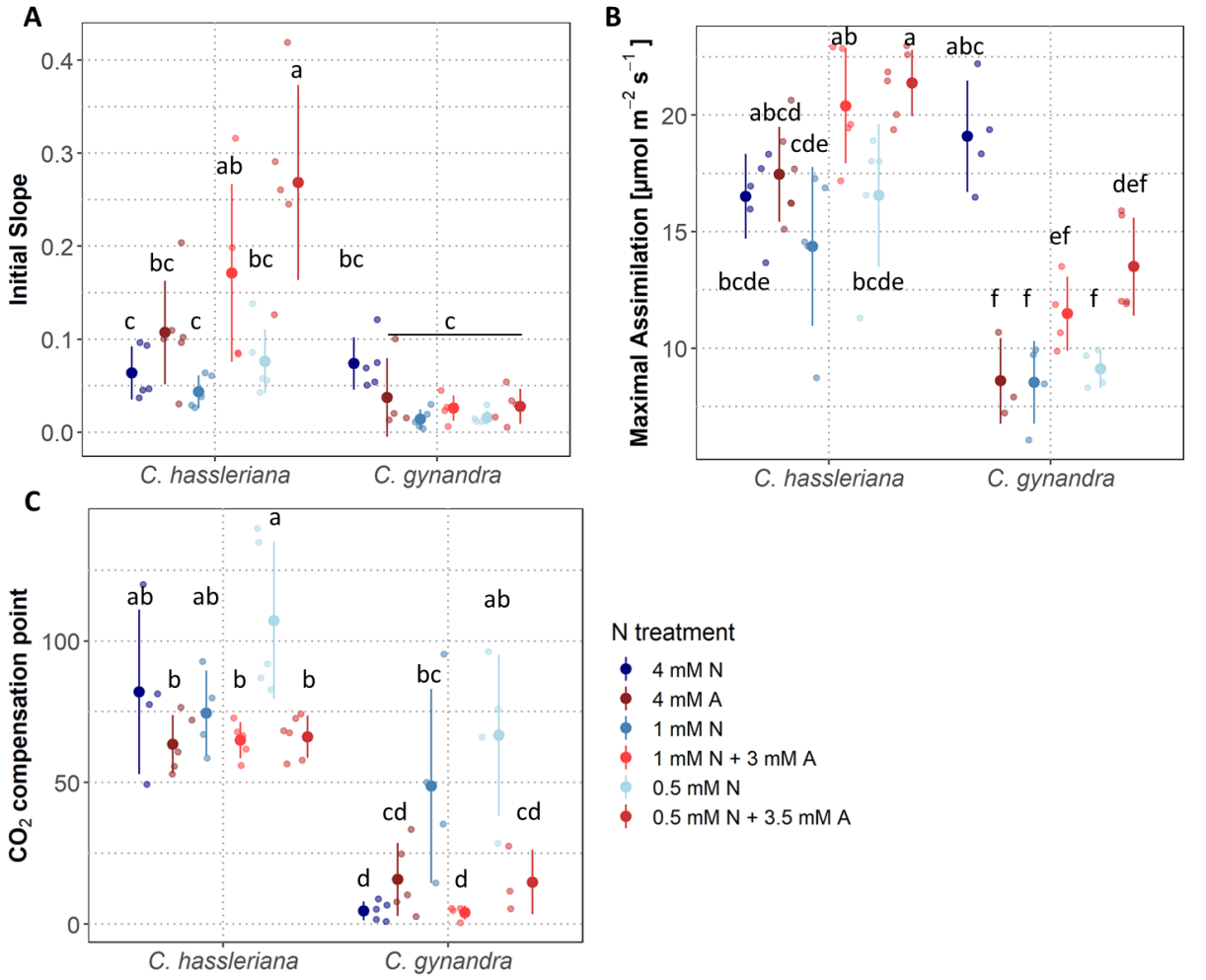

Figure 23: Comparative gas exchange measurements revealed a significant reduction of the photosynthesis rate in C_4 but not in C_3 species in response to ammonium treatment. Initial slopes (A), maximal assimilation (B) and CO_2 compensation points (C) of *C. hassleriana* (C_3) and *C. gynandra* (C_4) plants grown on Hoagland solution with different compositions of nitrate (N) and ammonium (A) including (1) nitrate only (4 mM N), (2) ammonium only (4 mM A), N deficiency (3) 0.5 mM N and a treatment substituted with ammonium (4) 0.5 mM N+ 3.5 mM A. In this and all following graphs the letters N and A indicate nitrate and ammonium, respectively. (n=5)

The C_3 species achieved the highest maximal CO_2 assimilation rates in treatments with a combination of both NO_3^- and NH_4^+ while in the C_4 species, the CO_2 assimilation rate was highest in the NO_3^--only treatment. However, NH_4^+ supplementation increased assimilation rates compared to the corresponding N deficiency treatment (Figure 23B). Initial slopes were highest in mixed treatments (Figure 23A). In the C_3 species, they were, however, not reduced in response to N deficiency unlike in the C_4 species. In *C. gynandra*, the initial slope was reduced in all treatments when compared to the full NO_3^- treatment (Figure 23A). While CO_2 compensation points of C_3 plants grown in nutrient solution containing NH_4^+ were lower than in plants not treated with NH_4^+, this reduction was not significant. In

contrast, significantly increased CO_2 compensation points were observed for C_4 plants grown in low N conditions but not in response to any NH_4^+ treatments (Figure 23C). Overall these results show that the photosynthetic efficiency of C_3 and C_4 *Cleome* species is differently affected by supplementation with ammonium.

Previously, all *Brassicaceae* species tested in the ammonium gradient experiment were able to grow in any combination of NH_4^+ and NO_3^- nutrient solution as well as in nutrient solution in which NH_4^+ was the only N source. The experiment further showed that most species grew best in nutrient solution containing a combination of both NH_4^+ and NO_3^-. This suggests that the plants can use NH_4^+ as an alternative N source to some extent. In contrast, the IRGA measurements demonstrated that ammonium negatively affected the photosynthetic efficiency of the C_4 *Cleome* species and only partially reversed the reduction of photosynthetic efficiency due to N deficiency while improving the photosynthetic performance of the C_3 species. To allow for direct comparison of the effects of N deficiency and NH_4^+ toxicity the biomass of plants grown in various compositions of NH_4^+ and NO_3^- (see above) was measured after 2 weeks of treatment (Figure 24).

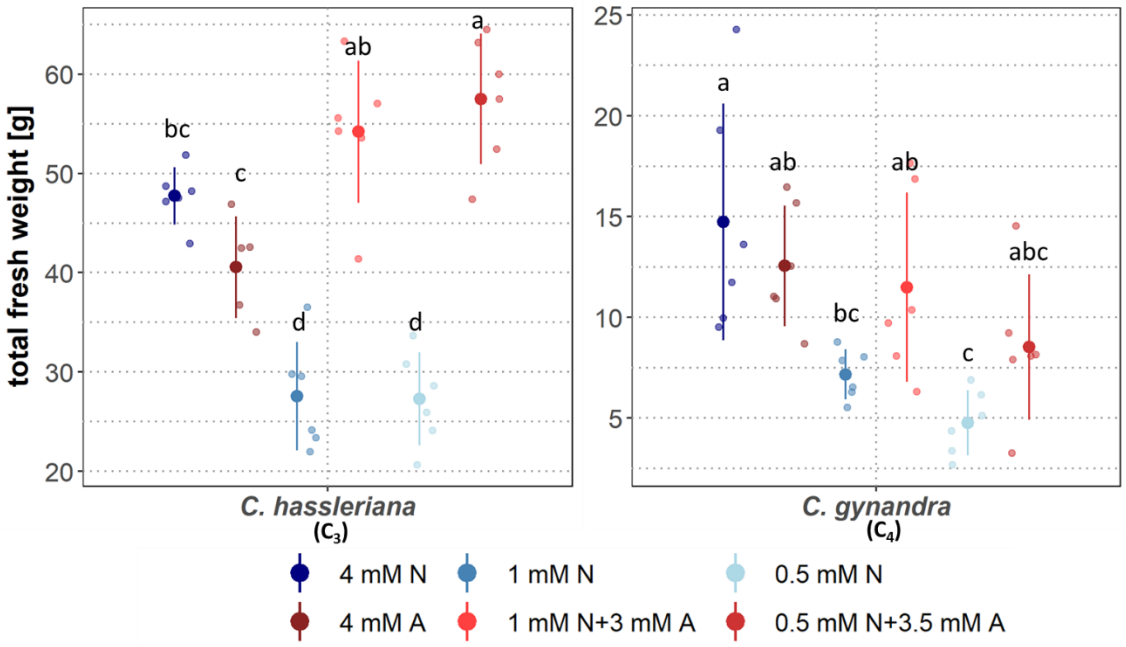

Figure 24: Biomass of *C. hassleriana* (C_3) and *C. gynandra* (C_4) grown in nutrient solution with different compositions of nitrate and ammonium as nitrogen sources. Plants were bottom watered with Hoagland solution with different compositions including (1) nitrate only (4 mM N), (2) ammonium only (4 mM A), two levels of N deficiency (3) 1 mM N, (5) 0.5 mM N and two treatments substituted with ammonium to reach a combined concentration of 4 mM: (4) 1 mM N + 3 mM A, (6) 0.5 mM N + 3.5 mM A. Biomass was measured after 2 weeks. (n=6)

The biomass of plants grown in nutrient solution containing only NH_4^+ was slightly but not significantly reduced compared to those grown in the NO_3^--only nutrient solution in both species. Furthermore, both species showed a significant decrease in biomass in response to the two N deficiency conditions (Figure 24). Additional NH_4^+ in the nutrient solution (treatment 4 and 6) significantly increased the total

fresh weight of the C_3 but not the C_4 species. As observed in previous experiments, the C_3 species achieved the highest biomass when watered with nutrient solution containing both NO_3^- and NH_4^+.

To further investigate these differences in biomass between the species anion content in roots and shoots was measured using IC (Figure 25). In the C_3 species *C. hassleriana,* NO_3^- content was significantly reduced in both roots and shoots in response to N deficiency as well as in the treatments containing ammonium in the nutrient solution (Figure 25A).

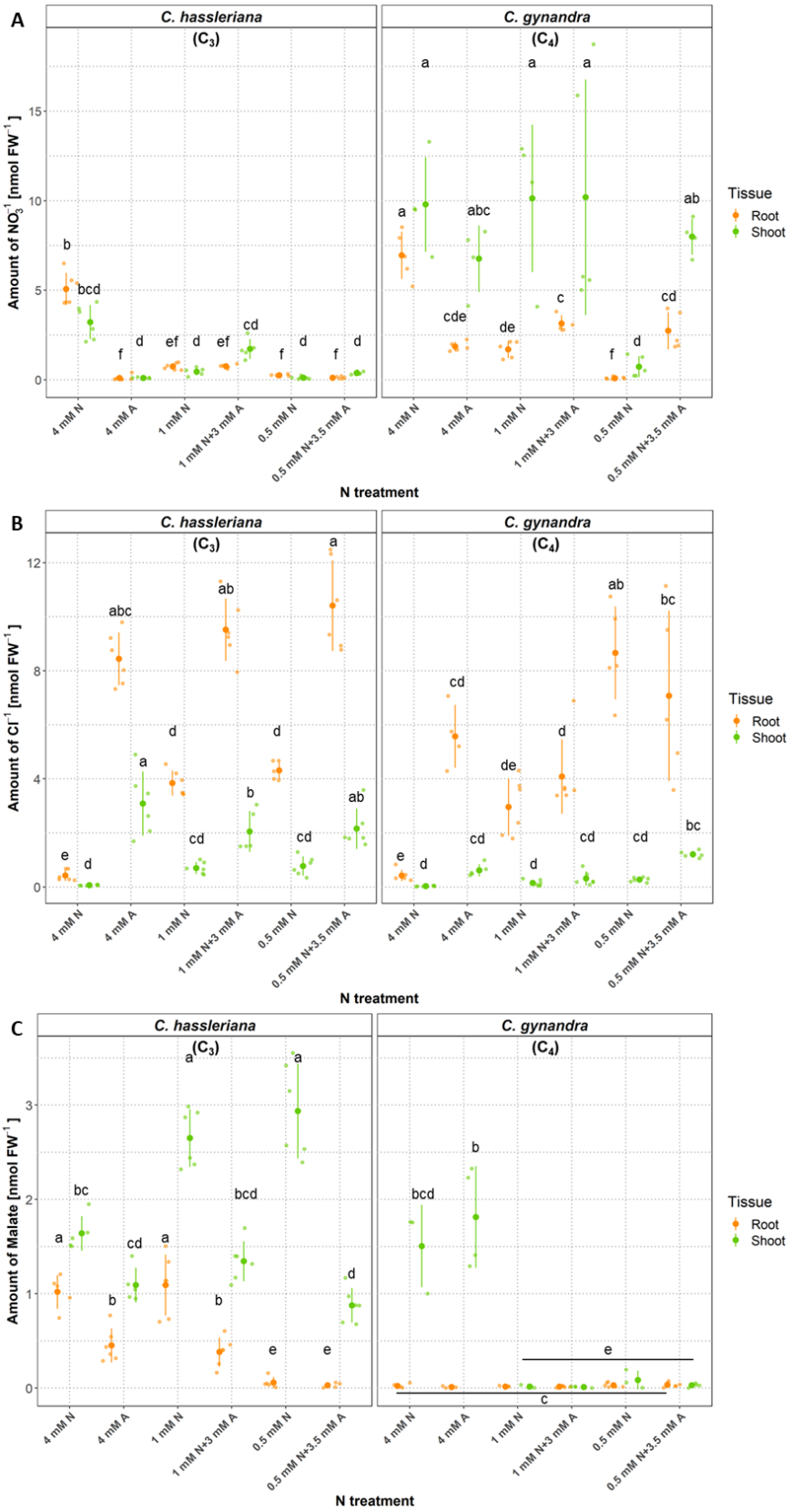

Figure 25: Anion content of *C. hassleriana* (C_3) and *C. gynandra* (C_4) grown in nutrient solution with different compositions of nitrate and ammonium as nitrogen sources. Plants were bottom watered with Hoagland solution with different compositions including (1) nitrate only (4 mM N), (2) ammonium only (4 mM A), two levels of N deficiency (3) 1 mM N, (5) 0.5 mM N and two treatments substituted with ammonium to reach a combined concentration of 4 mM: (4) 1 mM+ 3 mM A, (6) 0.5 mM N+ 3.5 mM A. Samples for measuring nitrate (A), chloride (B) and malate (C) content were taken after two weeks. (n=5)

In contrast, in the C_4 species NO_3^- levels decreased in roots in all treatments but only changed significantly in shoots in response to the strongest N deficiency treatment. The C_4 roots showed a clear increase in NO_3^- content in both mixed treatments when compared to the respective N deficiency treatment (Figure 25A). In both species, chloride accumulated mainly in roots and increased in low N conditions (Figure 25B). Chloride content further increased with increasing NH_4^+ in the nutrient solution. The increase between the low N treatments and their respective combined treatment was much more pronounced in the C_3 species (Figure 25B). Malate levels in the shoots of the C_3 species increased in N deficiency conditions but decreased in all A treatments (Figure 25C).

RuBisCO is the most abundant enzyme in plants making up 5 to 40 % of the total protein content depending on the species. Due to their improved CO_2 assimilation rates, C_4 species generally have a reduced demand for RuBisCO and therefore nitrogen. To test whether this is also the case in the *Cleome* genus total soluble protein content was measured using the Bradford method. Analysis of the total soluble protein content of the two *Cleome* species showed that the C_3 species had an overall higher protein content in leaves. In contrast, root protein content was similar in both species and was significantly reduced in both N deficiency treatments (Figure 26).

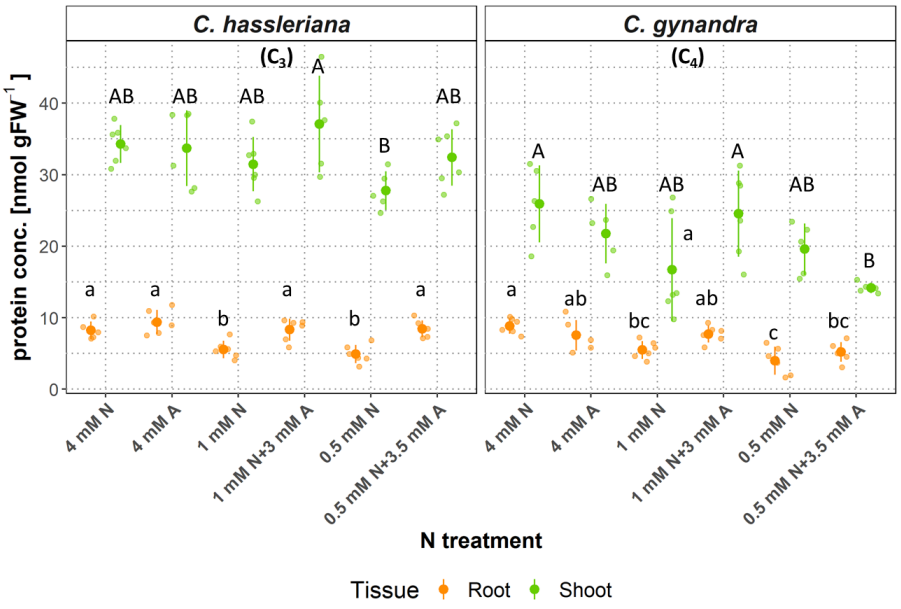

Figure 26: Total soluble protein concentration of *C. hassleriana* (C_3) and *C. gynandra* (C_4) grown in nutrient solution with different compositions of nitrate and ammonium as nitrogen sources. Plants were bottom watered with Hoagland solution with different compositions including (1) nitrate only (4 mM N), (2) ammonium only (4 mM A), two levels of N deficiency (3) 1 mM N, (5) 0.5 mM N and two treatments substituted with ammonium to reach a combined concentration of 4 mM: (4) 1 mM+ 3 mM A, (6) 0.5 mM N+ 3.5 mM A. Protein content was measured after 2 weeks of treatment. (n=6)

4.2.5. Effects of ammonium supplementation vary between C_3 and C_3C_4-intermediate species

To see whether the positive effect on biomass in plants substituted with ammonium observed in the C_3 species *C. hassleriana* is conserved in C_3 species of other genera the experiment was repeated with the C_3- and C_3C_4-intermediate species from the *Moricandia* and *Diplotaxis* genus used in the previous experiments. After two weeks of treatment, the biomass of the two C_3C_4-intermediate *Diplotaxis* species showed a significant decrease in both N deficiency treatments and all NH_4^+ treatments compared to the medium containing only NO_3^-, while the fresh weight of *D. viminea* (C_3) was only significantly reduced in response to strong N deficiency (0.5 mM N). The biomass of the intermediates in the mixed treatments (4,6) was, however, significantly higher than in the full NH_4^+ and strong N deficiency treatment. (Figure 27A).

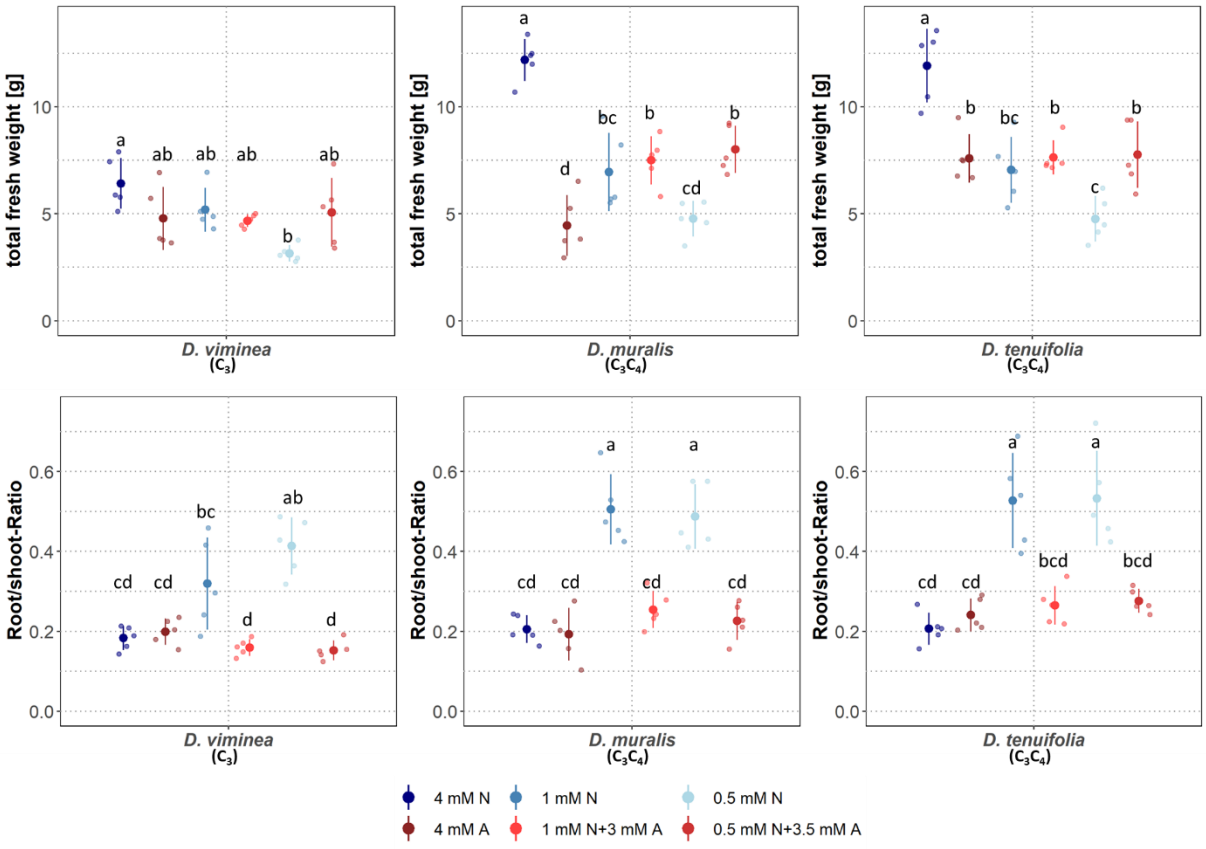

Figure 27: Biomass of *D. viminea* (C_3), *D. muralis* (C_3C_4) and *D. tenuifolia* (C_3C_4) grown in nutrient solution with different compositions of nitrate and ammonium as nitrogen sources. Plants were bottom watered with Hoagland solution with different compositions including (1) nitrate only (4 mM N), (2) ammonium only (4 mM A), two levels of N deficiency (3) 1 mM N, (5) 0.5 mM N and two treatments substituted with ammonium to reach a combined concentration of 4 mM: (4) 1 mM N + 3 mM A), (6) 0.5 mM N+ 3.5 mM A. Biomass was measured after 2 weeks. (n=6)

Analysis of Root/Shoot-Ratios further revealed that the Root/Shoot-Ratio was increased significantly in both N deficiency treatments in the intermediates and the strongest deficiency treatment in the C_3 species (Figure 27B).

In the *Moricandia* genus changes in biomass varied between treatments and species (Figure 28A).

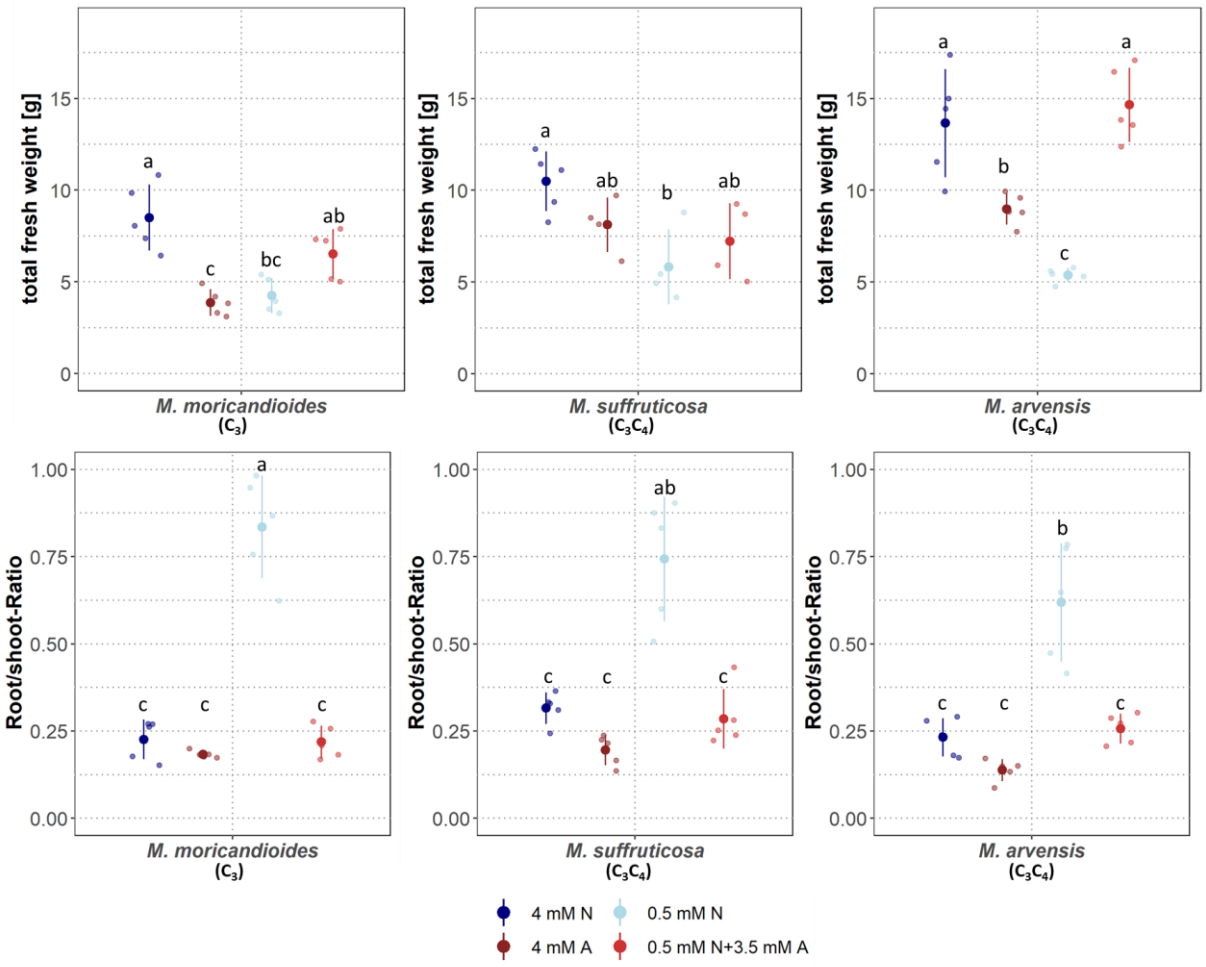

Figure 28: Biomass (A) and Root/Shoot-Ratio of *M. moricandioides* (C_3), *M. suffruticosa* (C_3C_4) and *M. arvensis* (C_3C_4) grown in nutrient solution with different compositions of nitrate and ammonium as nitrogen sources. Plants were bottom watered with Hoagland solution with different compositions including (1) nitrate only (4 mM N), (2) ammonium only (4 mM A), N deficiency (3) 0.5 mM N and a treatment substituted with ammonium (4) 0.5 mM N+ 3.5 mM A. Biomass was measured after 2 weeks. (n=6)

The C_3 species *M. moricandioides* showed a significant reduction in biomass when grown with NH_4^+ as the only N source. Under N deficiency conditions (0.5 mM) biomass and Root/Shoot-Ratio were significantly decreased and increased, respectively (Figure 28A+B). While supplementation with NH_4^+ led to a reduction of the Root/Shoot-Ratio to levels of plants grown on standard Hoagland medium (4 mM N), the biomass was not fully restored to control levels. While both intermediates showed a reduction in biomass in the NH_4^+ only and N deficiency treatment, both changes were only significant in *D. tenuifolia.* Similarly, while an increase of biomass was observed for both species in the mixed treatment, only *D. tenuifolia* reached control levels (Figure 28A). Both intermediates showed an

increased Root/Shoot-Ratio only under N deficiency conditions, which was also illustrated in the C_3 species. The change in Root/Shoot-Ratio was highest in the C_3 species and gradually decreased with decreasing CO_2 compensation points (Figure 28B).

As observed in *C. hassleriana*, the C_3 species of the *Moricandia* and *Diplotaxis* genera both showed a decrease in soluble protein content under low N conditions as well as an increase in both shoots and roots in the combined treatments when compared to the lowest N deficiency treatment (Supplemental Figure 3). The intermediates however varied in their responses. In the *Diplotaxis* intermediates soluble protein content was only increased in combined treatment and this increase was reduced in the intermediate species with a lower CO_2 compensation point (*D. tenuifolia*) (Supplemental Figure 3). The two *Moricandia* intermediates showed an increase of soluble protein content in the combined treatments only in roots and a decrease of soluble protein content in response to N deficiency only in shoots (Supplemental Figure 3).

4.3. N uptake and assimilation in C_3 and C_4 *Cleome* species

Changes in anion contents observed in previous experiments indicate that uptake and distribution of N play a key role in conferring the C_4 plant's improved NUE and tolerance to N deficiency. Higher N uptake abilities as well as more efficient distribution of assimilated N might explain these differences. Therefore, the N uptake ability of C_3 and C_4 *Cleome* species under N deficiency conditions was investigated via a re-supply experiment.

4.3.1. Quantification of anion content after N-resupply indicates faster uptake of newly available N in C_4 species as well as differences in the response to NO_3^- and NH_4^+

To study the ability of C_3 and C_4 species to recover from N deficiency, C_3 and C_4 *Cleome* species were grown in 1 mM nitrate medium for two weeks before being transferred to a full medium (4 mM nitrate). The speed of their response to newly available nitrate was assessed by taking samples 30 min, 60 min, 120 min and 240 min after transfer to 4 mM medium. Anion content was measured using IC and compared to that of plants that remained in the 1 mM medium (0 min). The distribution of nitrate between the roots and shoots differed between species, with the C_4 species accumulating more nitrate in the roots compared to the C_3 species, in which nitrate was more abundant in the shoots. In *C. hassleriana* the nitrate content did not change within the 4 h timeframe (Figure 30A).

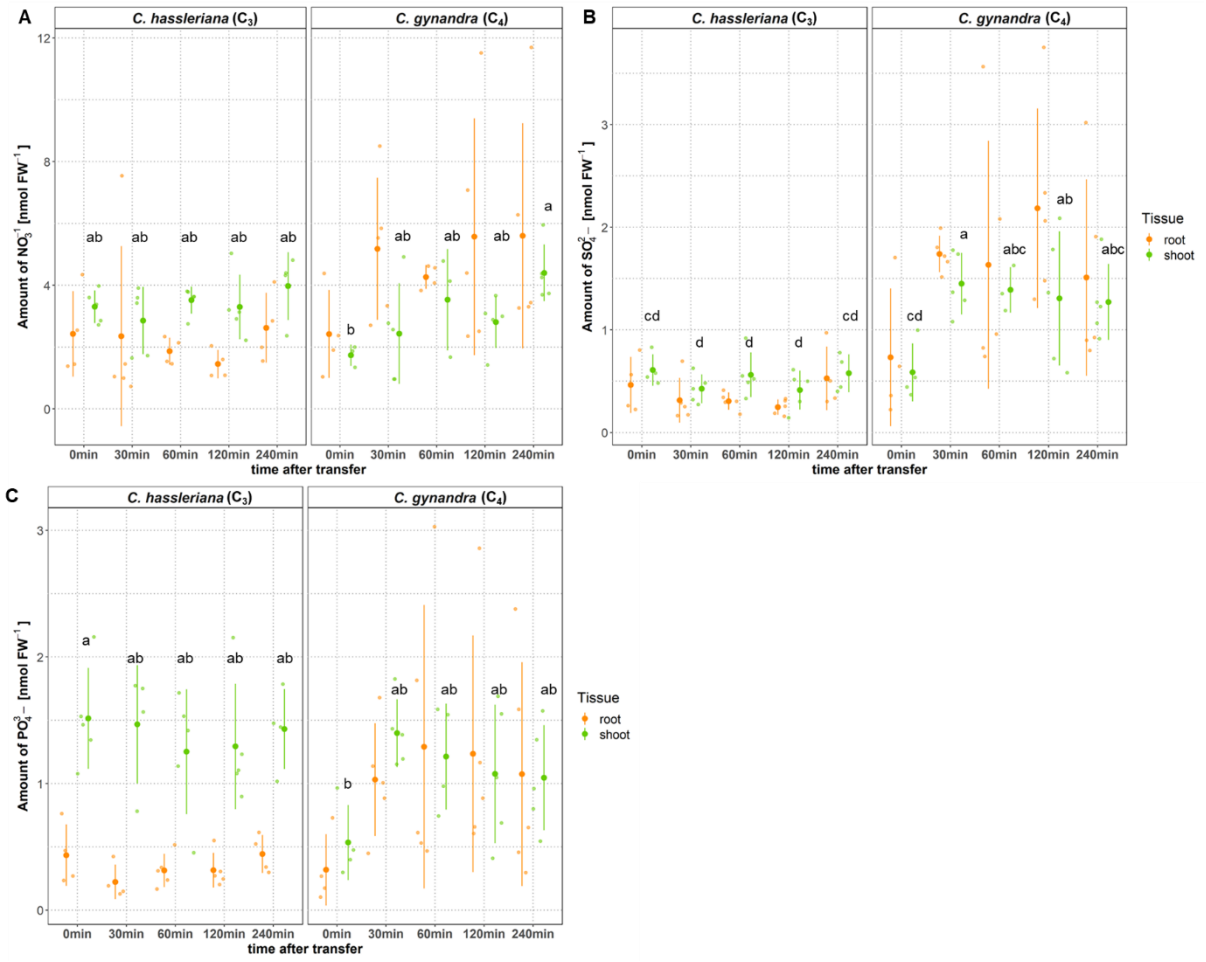

Figure 29: Anion content of *C. hassleriana* (C_3) and *C. gynandra* (C_4) after re-supply of nitrate. Plants were bottom watered with 1 mM Hoagland solution for 2 weeks. Samples for measuring nitrate (A), phosphate (B) and sulphate (C) content were taken 30 min, 60 min, 120 min and 240 min after transfer to 4 mM nitrate nutrient solution. (n=5)

In contrast, the nitrate content in both roots and shoots in *C. gynandra* already increased after 30 min, however, due to high variation especially in the roots this change was not significant. Despite this variation, a significant increase of nitrate in the shoots was observed after 240 min (Figure 29A). Similar to the content of nitrate measured, the sulphate content increased significantly in shoots of C_4 *Cleome* plants but remained the same in the C_3 species (Figure 29B). Finally, the phosphate content was also unaffected by the re-supply with nitrate in the C_3 species (Figure 29C). However, unlike nitrate and sulphate, phosphate content was higher in the shoots compared to the roots of *C. hassleriana*. In the C_4 species, the sulphate amount in both roots and shoots increased after 30 min but due to high variation, this increase was not significant.

In previous experiments, it was shown that both species were able to use both nitrate and ammonium as N sources. The optimal amount of ammonium varied between C_4 and C_3 *Cleome*. The findings indicate a higher NH_4^+ tolerance of C_3 plants but also point to a better NH_4^+ uptake and assimilation ability of the C_3 species. To test this hypothesis, C_3 and C_4 *Cleome* plants were grown under deficiency

conditions (1 mM nitrate) for two weeks before being provided with a sufficient concentration of nitrate or ammonium, respectively. The speed of their response to newly available nitrogen sources was again investigated via a time course experiment. Samples were taken 1 h, 4 h and 24 h after the transfer to either full nitrate (4 mM N) or full ammonium (4 mM A) medium. Anion content was measured using IC and compared to plants remaining in the N deficiency medium (contr). Nitrate content in leaves of the C_4 and C_3 plants increased after 1 h and 4 h, respectively (Figure 30).

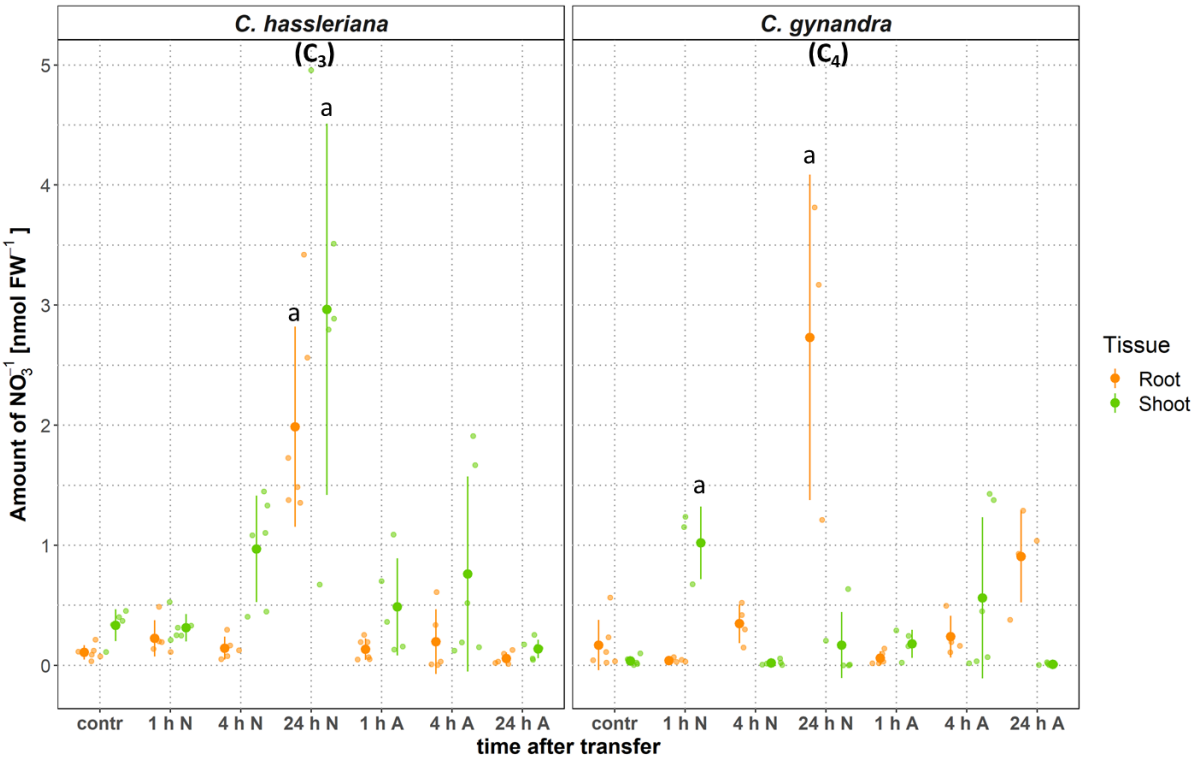

Figure 30: Nitrate content of *C. hassleriana* (C_3) and *C. gynandra* (C_4) after re-supply with nitrate or ammonium. Plants were bottom watered with Hoagland solution 1 mM for 2 weeks. Samples for measuring nitrate content were taken 1 h, 4 h and 24 h after transfer to either 4 mM nitrate or 4 mM ammonium nutrient solution. The letters A and N after the time points indicate ammonium and nitrate medium, respectively (n=5)

While the C_3 species showed a further increase in nitrate levels in both roots and shoots after 24 h, C_4 leaf nitrate levels dropped back to the level of the control condition, before increasing in roots after 24 h, which could indicate a fast turn-over of the nitrate into amino acids and proteins. Surprisingly, both C_3 and C_4 plants supplied with medium containing only NH_4^+ also showed increased nitrate content in leaves after 4 h and 1 h, respectively (Figure 30). This increase of NO_3^- after 24 h in roots of C_4 plants, could be caused by bacterial oxidation, by which NH_4^+ from the nutrient solution is converted into NO_3^- and taken up by the plant. This hypothesis was tested by eliminating potential bacterial contamination trough adding ampicillin to the nutrient solution (Figure 31).

Similar to the previous experiment, a significant increase of NO_3^- content in roots and shoots of the C_3 species was observed 24 h after the transfer to the N nutrient solution (Figure 31A).

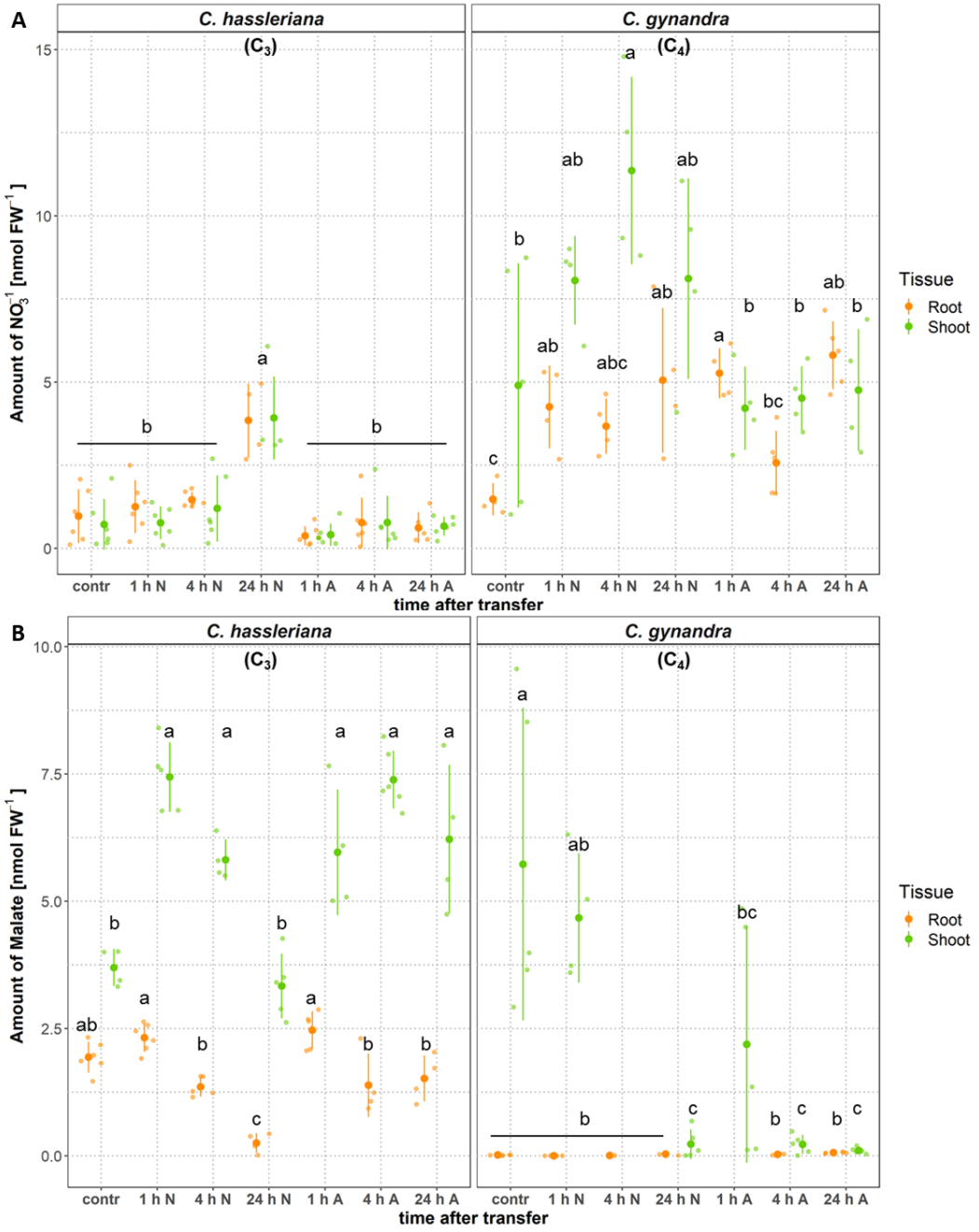

Figure 31: Anion content of *C. hassleriana* (C_3) and *C. gynandra* (C_4) after re-supply with nitrate or ammonium. Plants were bottom watered with Hoagland solution 1 mM for 2 weeks. Samples for measuring nitrate (A) and malate (B) content were taken 1 h, 4 h and 24 h after transfer to either 4 mM nitrate or 4 mM ammonium medium. The letters A and N after the time points indicate ammonium and nitrate medium, respectively. (n=5)

In contrast, plants transferred to A-nutrient solution instead did not show any significant changes in NO_3^- levels. C_4 roots on the other hand showed significantly higher amounts of NO_3^- after 1 h in both media (Figure 31A). They, however, exhibited differences in their reaction to the two N sources in relation to shoot nitrate content. While no changes were observed in the leaves of plants transferred

to the A medium, nitrate levels increased in shoots of plants resupplied with nitrate starting at 1 h after transfer, kept increasing at the 4 h time point but decreased back to the level observed after 1 h (Figure 31).

Analysis of anion content further revealed a clear difference in malate metabolism between the two *Cleome* species. In general malate content was higher in the C_3 species compared to the C_4 species, which contained almost no malate in roots (Figure 31B). In leaves of the C_3 species, the malate content increased after 1 h in both media. In response to the N medium, a decrease was observed 4 h after the transfer, and malate levels were back at control levels after 24 h. In contrast, shoot malate levels stayed high in plants supplied with A medium. In both nutrient solutions root malate content decreased, with a stronger decrease in N medium (Figure 31B). Overall there was a high variation in the measured malate levels in the C_4 species. Opposite to the C_3 species, a strong reduction of shoot malate content was observed in response to both media in the C_4 species, while this decrease was faster in the plants transferred to A medium (Figure 31B).

4.3.2. Quantification of ^{15}N in roots and shoots of C_3 and C_4 *Cleome* plants shows differences in uptake and distribution between NO_3^- and NH_4^+

Previous experiments indicate a faster uptake of and preference for nitrate in the C_4 species. Furthermore, the C_3 species were shown to better utilize provided ammonium. However, the mechanism causing these differences between the C_3 and C_4 species remains unclear. To gain further insights into the N uptake mechanism in both species, the uptake of ^{15}N-labeled potassium nitrate (KNO_3) and ammonium chloride (NH_4Cl) by N-deficient C_3 and C_4 *Cleome* plants was measured using GC-IRMS. Both species showed a strong uptake of N in roots when transferred to either N source (Figure 32).

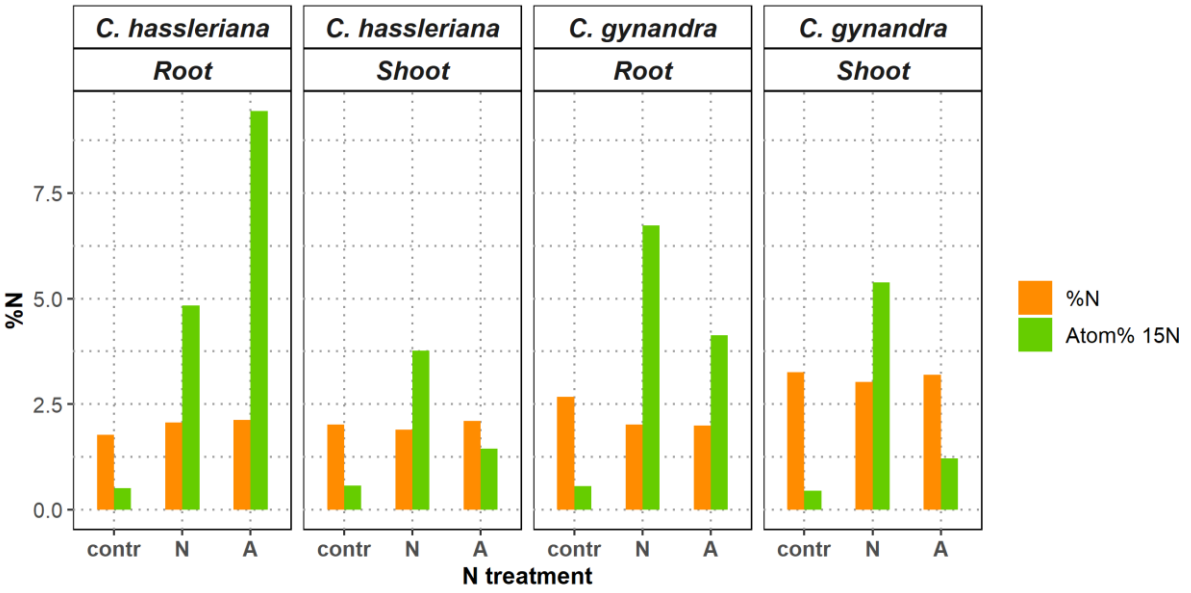

Figure 32: Uptake and distribution of ^{15}N differed between *C. hassleriana* (C_3) and *C. gynandra* (C_4) depending on the N source. Plants were bottom watered with 1 mM N Hoagland solution for 2 weeks before being transferred to $^{15}NO_3^-$ and $^{15}NH_4^+$ nutrient solution, respectively. Samples for measuring uptake of ^{15}N were taken 4 h after transfer to either nutrient solution. The plants in the control condition remained in the unlabelled 1 mM N nutrient solution. The letters A and N refer to ammonium and nitrate nutrient solutions, respectively. (n=1-3)

C_4 plants that were moved to nitrate-containing nutrient solution took up more N in their roots compared to their C_3 relatives. However, the uptake in the roots of the C_3 plants was almost 5 % higher when provided with ammonium instead of nitrate. In contrast, in the C_4 species, N uptake was higher in the nitrate-containing nutrient solution (~2 %), confirming the preference for nitrate displayed by the C_4 species. Similar to roots, uptake of N was higher in the shoots of C_4 plants provided with nitrate medium. In shoots of the ammonium-treated plants, however, comparatively lower N uptake rates were observed in both species.

4.3.3. Expression analysis of N assimilation genes in C_3 and C_4 *Cleome* species in the presence of NH_4^+

Analysis of nitrate content in C_3 and C_4 *Cleome* species grown in nutrient solutions with different compositions of nitrate and ammonium showed a higher accumulation of nitrate in shoots and roots of the C_4 species irrespective of treatment which could indicate a more efficient uptake or assimilation of nitrate in the C_4 species. This could be due to differences in the expression levels and pattern of nitrate reductases and nitrate transporter genes.

In the model organism *A. thaliana* nitrate assimilation is performed by a minor (AtNIA1) and major (AtNIA2) nitrate reductase isoform. BLAST was used to identify peptide sequences of various NR orthologs in the two *Cleome* species as well as other species known to have multiple NR isoforms

(*Brassica napus*, *Brassica rapa* and *Zea mays)*. These sequences were used to build a phylogenetic tree (Figure 33).

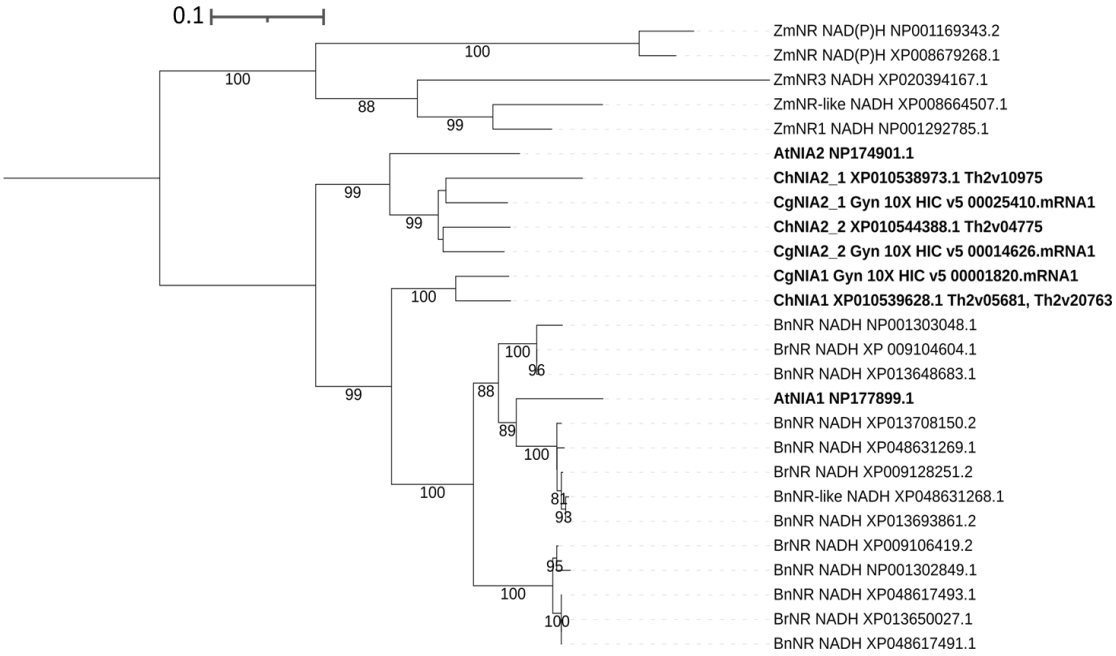

Figure 33: Phylogenetic analysis of MSA of peptide sequences of nitrate reductase (NR) encoding genes from *Z. mays* and various *Brassicaceae* species including the C_4 species *C. gynandra.* The MSA was build using the MAFFT online tool. Phylogenetic tree was determined using the Maximum Likelihood method (100 bootstraps).

According to this phylogenetic analysis one ortholog of *AtNIA1* and two orthologs of *AtNIA2* were identified in each *C. hassleriana* and *C. gynandra*. The two orthologs will be referred to as CxNIA2-1 and CxNIA2-2, respectively.

Expression analysis of the two *NIA2* isoforms was performed using qPCR and revealed clear differences between the C_3 and C_4 species in expression level and also in their response between the different N conditions (Figure 34A+B). Overall both isoforms were more highly expressed in shoots in both species. In the C_3 species, root and shoot expression of both isoforms was highest under full N conditions and strongly reduced in all other conditions. In contrast, *CgNIA2-1* was most highly expressed in roots under N deficiency (1 mM) und in shoots in the combined treatment (1 mM N+3 mM A) (Figure 34A). While *CgNIA2-2* showed a similar expression pattern in the roots, expression in shoots was highest in 1 mM N treatment, followed by the combined treatment (Figure 34B).

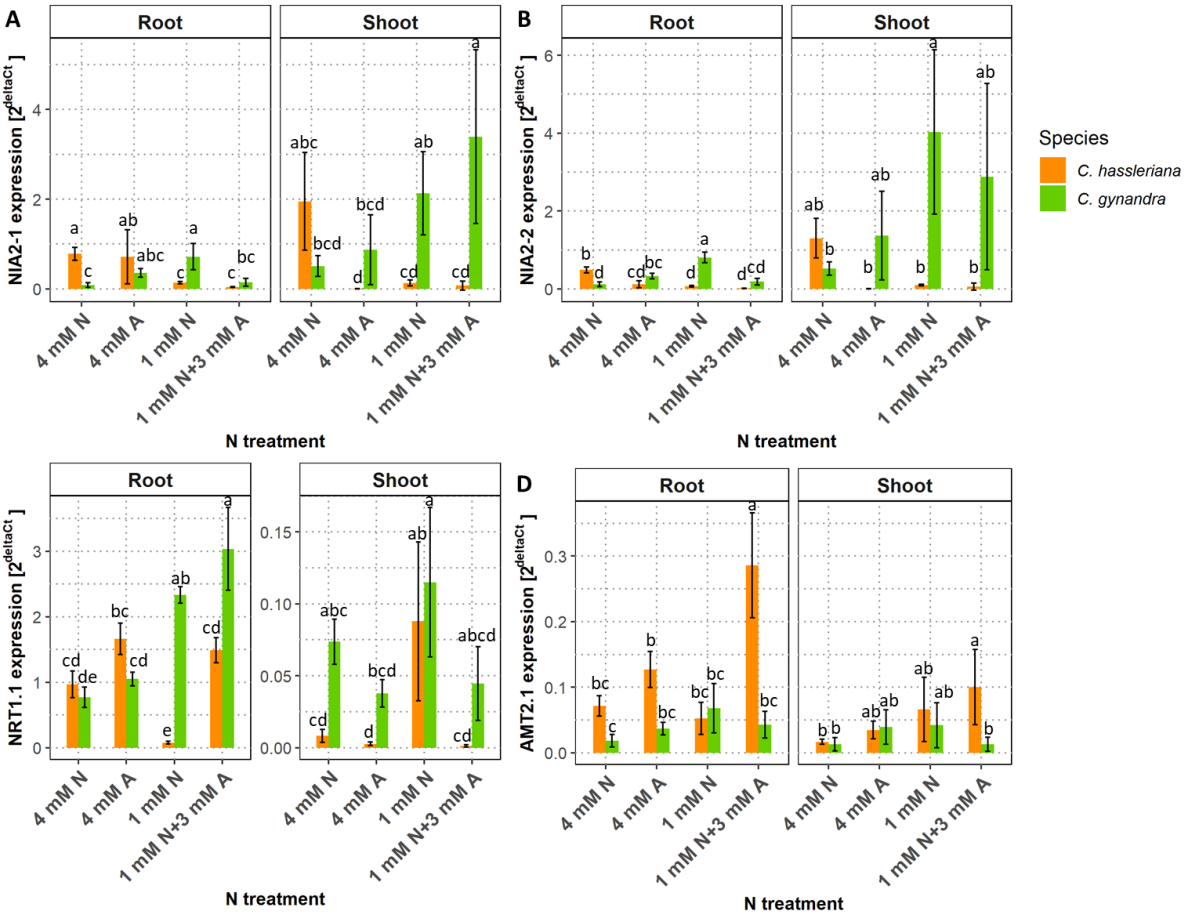

Figure 34: Relative expression of genes involved in nitrogen assimilation in roots and shoots of *C. hassleriana* (C_3) and *C. gynandra* (C_4) grown in nutrient solution with different compositions of nitrate and ammonium as nitrogen sources. Plants were bottom watered with Hoagland solution with different compositions including (1) nitrate only (4 mM N), (2) ammonium only (4 mM A), N deficiency (3) 1 mM N and a treatment substituted with ammonium (4) 1 mM N + 3.5 mM A. Relative gene expression ($2^{-\Delta Ct}$) displayed in log2 was determined by qRT-PCR. The housekeeping gene *ACT1* was used. (n=3)

The C_3 and C_4 *Cleome* species also showed differences in their nitrate uptake ability and ammonium tolerance which indicates that there might also be differences in the expression of nitrate and ammonium transporters. Therefore, the expression of genes encoding a nitrate (NRT1.1) and an ammonium (AMT2.1) transporter was also quantified. *NRT1.1* was more highly expressed in roots in both species (Figure 34C). Surprisingly, both species showed similar expression levels in nitrate (4 mM N) and ammonium (4 mM A) nutrient solution with the expression being slightly higher in the C_3 species in both conditions. The two species, however, showed significant differences in expression levels under N deficiency and in response to the combined medium. Under N deficiency conditions (1 mM N), expression of *NRT1.1* was significantly reduced and increased in the C_3 and C_4 species, respectively. In Figure 34C, in the combined nutrient solution expression of *NRT1.1* was at a similar level to both the nitrate and ammonium medium in the C_3 species. In contrast, in the C_4 species expression was significantly higher compared to the expression level in both the other treatments as well as in the C_3 species in the same treatment.

In both full nitrate and ammonium conditions, expression of *AMT2.1* was higher in the roots of the C_3 species (Figure 34D). While expression in the C_4 species did not change between treatments, *AMT2.1* expression increased significantly in response to the combined treatment. In shoots, *AMT2.1* was most highly expressed under N deficiency conditions in the C_3 species.

4.3.4. Nitrate reductase activity is increased in the roots of C_4 species in the presence of NH_4^+

Expression analysis showed surprising differences in the expression of NR encoding genes *NIA2-1* and *NIA2-2* between the C_3 and C_4 Cleome species but also in response to ammonium medium. However, due to post-translational regulation of the NR enzyme, the expression data might not accurately reflect its activity. Therefore, the specific activity in various N treatments was determined through quantification of the produced nitrite using sulphanilamide and N-(1-Naphthyl)-ethylenediamine (NED).

Analysis of the NR activity revealed clear differences between the C_3 and C_4 species. In the C_3 species, the NR activity was comparable in shoots and roots and did not vary strongly between treatments (Figure 35).

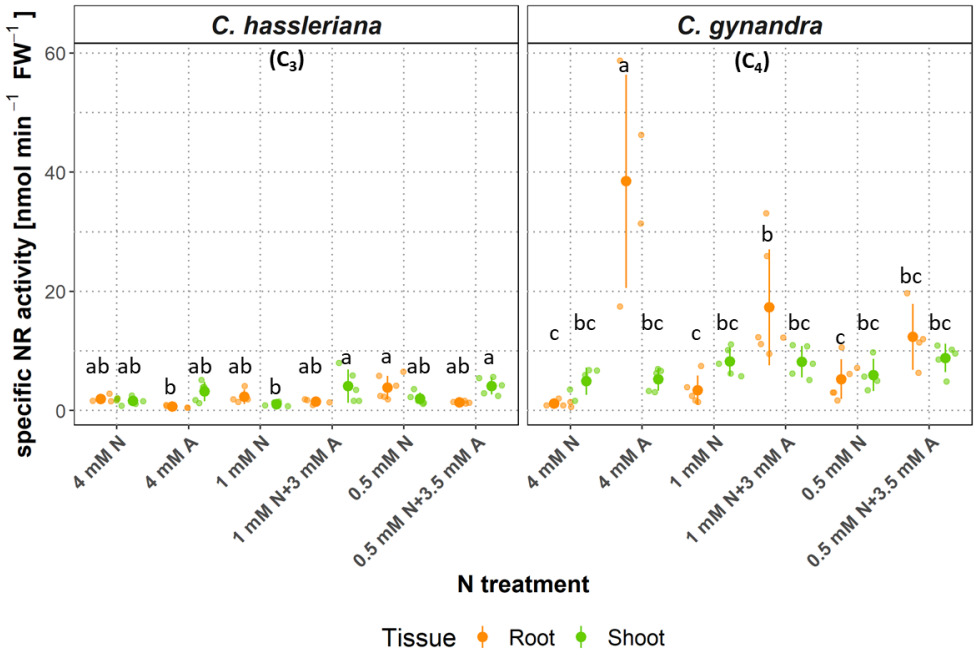

Figure 35: Nitrate reductase (NR) activity of *C. hassleriana* (C_3) and *C. gynandra* (C_4) grown in media with different compositions of nitrate and ammonium as nitrogen sources. Plants were bottom watered with Hoagland solution with different compositions including (1) nitrate only (4 mM N), (2) ammonium only (4 mM A), two levels of N deficiency (3) 1 mM N, (5) 0.5 mM N and two treatments substituted with ammonium to reach a combined concentration of 4 mM: (4) 1 mM+ 3 mM A), (6) 0.5 mM N+ 3.5 mM A. In this and all following graphs the letters N and A indicate nitrate and ammonium, respectively. Nitrate reductase activity was measured after 2 weeks. (n=6)

Similarly, in the C_4 species, the NR activity was also not significantly different between roots and shoots in all treatments containing only nitrate (4 mM, 1 mM and 0.5 mM N) and did not change under N deficiency conditions (1 mM and 0.5 mM N). However, in treatments containing ammonium a strong increase in NR activity was observed in the roots (4 mM and 1 mM). This increase in activity seemed to be dose-dependent as the rise was stronger with higher ammonium concentration in the nutrient solution.

4.3.5. Analysis of protein and amino acid content after N re-supply confirms nitrate preference and reveals lower *de novo* synthesis rates of amino acids in C_4 species

The potential difference in protein turnover rates depending on the N source was investigated by measuring the total soluble protein concentration in N-deficient C_3 and C_4 *Cleome* plants after re-supply with either NO_3^- or NH_4^+. In general protein content was higher in shoots compared to roots. Soluble protein concentrations were significantly reduced in plants grown under low N conditions (1 mM N) in both species (Figure 36). Protein levels did not increase in either species within 24 h upon re-supply with either nitrate or ammonium. In the C_3 species, shoot protein concentrations decreased even further after re-supplying with nitrate but not ammonium.

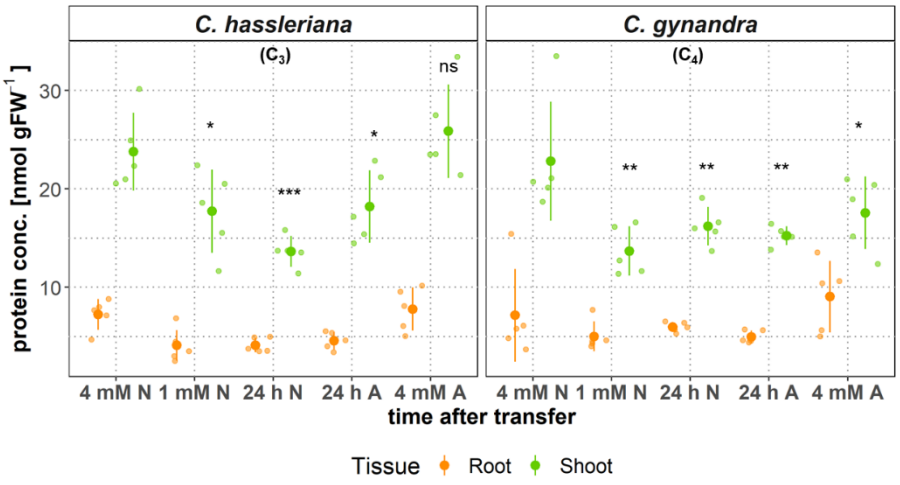

Figure 36: Comparison of soluble protein concentration in roots and shoots of *C. hassleriana* (C_3) and *C. gynandra* (C_4) grown in N deficiency medium before and after re-supply with nitrate. Plants were bottom watered with 1 mM Hoagland solution for 2 weeks. Samples for measuring protein content were taken 24 h after transfer to either 4 mM nitrate (24 h N) or 4 mM ammonium (24 h A) nutrient solution. Furthermore, samples from plants grown in full nitrate (4 mM N) and full ammonium (4 mM A) were used for comparison. Pairwise T-Test was performed comparing each treatment to the full nitrate (4 mM N) treatment. Significance levels are indicated by asterisks (* $p<0.05$; ** $p<0.001$; *** $p<0.0001$). (n=5)

In the C_3 species, protein concentrations were similar in plants grown in full N and A medium. In contrast, in the C_4 species soluble the protein content was significantly lower in the ammonium treatment in leaves but showed a slight increase in the roots (Figure 36).

Total soluble protein concentration was also measured as described above, in the shoots and roots of plants grown in nutrient solution supplemented with 4 mM ampicillin. Like in the previous experiment, a higher amount of soluble protein was observed in the leaves of the C_3 species (Figure 36,37).

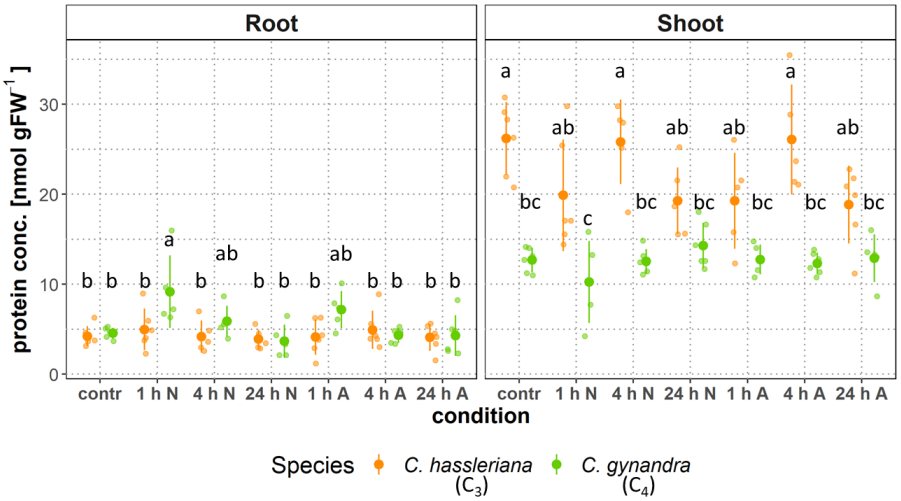

Figure 37: Total soluble protein concentration of *C. hassleriana* (C_3) and *C. gynandra* (C_4) after re-supply with nitrate or ammonium. Plants were bottom watered with 1 mM Hoagland solution containing ampicillin for 2 weeks. Samples for measuring protein concentration were taken 1 h, 4 h and 24 h after transfer to either 4 mM nitrate or 4 mM ammonium nutrient solution, which also contained ampicillin. The letters A and N after the time points indicate ammonium and nitrate nutrient solutions, respectively. (n=6)

While there were slight changes in the protein concentration in between the treatments in the shoots, no significant differences in protein content were observed in the C_3 species in either roots or shoots. In the C_4 species, however, root protein levels increased after 1 h in both media and then decreased back to control conditions (Figure 37). Moreover, after 24 h the protein concentration was slightly albeit not significantly higher in the plants grown on nitrate compared to ammonium.

Metabolite profiling revealed a significant difference in overall amino acid content between the C_3 and C_4 *Cleome* species. Most amino acids were more abundant in the C_4 species, especially in leaves in control and low N conditions. Taken together with the lower protein content observed in C_4 shoots this could suggest that amino acids might act as nitrogen storage compounds in the C_4 species. The protein content in the C_4 species also differed between plants grown in nitrate and ammonium medium, respectively. Ammonium medium seemed to reduce the total protein amount in the C_4 but not the C_3 *Cleome* species. To understand the differences in the regulation of the amino acid pools between the C_3 and C_4 species, N-deficient plants were re-supplied with N in the form of ^{15}N-labeled potassium nitrate (KNO_3) and ammonium chloride (NH_4Cl), respectively. The incorporation of ^{15}N into

the different amino acids was determined by quantifying the percentage of m_{+1}-fragments for each amino acid as a proxy for *de novo* synthesis in samples taken 24 h and 48 h after supplying the plants with ^{15}N medium.

Ammonium from various origins, including uptake via the roots, produced from nitrate reduction or photorespiration, is assimilated into glutamine and glutamate via the GS/GOGAT-cycle. Gln and Glu subsequently act as N donors for the biosynthesis of other amino acids (Miflin and Habash, 2002). Glutamate is, therefore, an important indicator of the N status of the plant. The analysis of the incorporation of ^{15}N-labeled nitrate (N) and ammonium (A) into amino acids by N-deficient plants revealed significant differences in the amount of *de novo* synthesized amino acids between the C_3 and C_4 species, depending on the N source. Levels of newly produced Glutamate (Glu) and Proline (Pro), which is derived from the Glu, are slightly but significantly higher in the C_3 compared to the C_4 species in both leaves and roots 24 h after re-supply with N (Figure 38).

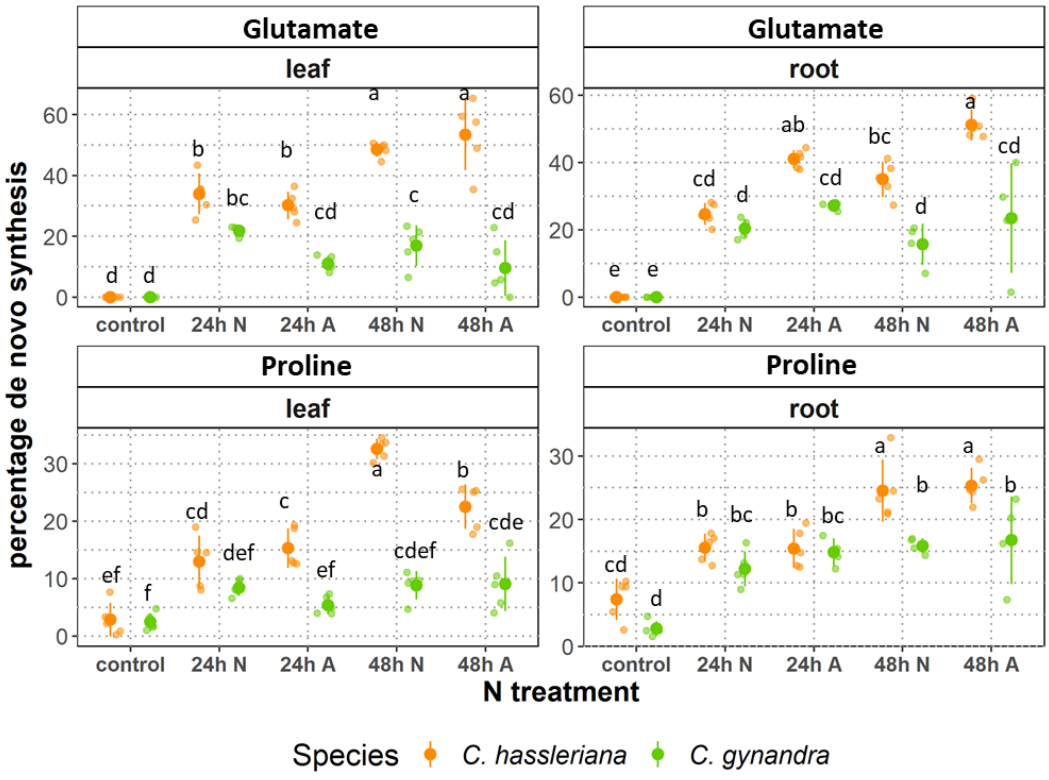

Figure 38: Percentage of *de novo* synthesized glutamate and proline in leaves and roots of *C. hassleriana* (C_3) and *C. gynandra* (C_4) after re-supply with nitrate or ammonium. Plants were bottom watered with 1 mM Hoagland solution for 2 weeks before being transferred to $^{15}NO_3^-$ and $^{15}NH_4^+$ nutrient solution respectively. Samples for measuring incorporation of ^{15}N into amino acids were taken 24 h and 48 h after transfer to either nutrient solution. The letters A and N after the timepoints indicate ammonium and nitrate nutrient solution, respectively. (n=4)

However, in plants provided with ammonium *de novo* synthesis of Glu and Pro in leaves was significantly lower in the C_4 species. No further production of Glu and Pro was observed after 48 h with either N sources in the C_4 species and the percentage of ^{15}N-labeled Glu was even slightly reduced. In

contrast, in the C_3 species the percentage of newly produced Glu and Pro further increased in both N treatments. There also seemed to be N-source-dependent differences in amino acid production between leaves and roots. While the N source did not affect Glu leaf levels and Pro root levels, Pro leaf and Glu root content was reduced and increased, respectively, when supplied with ammonium (Figure 38).

The photorespiratory amino acids serine (Ser) and glycine (Gly) shared a pattern across treatments (Figure 39).

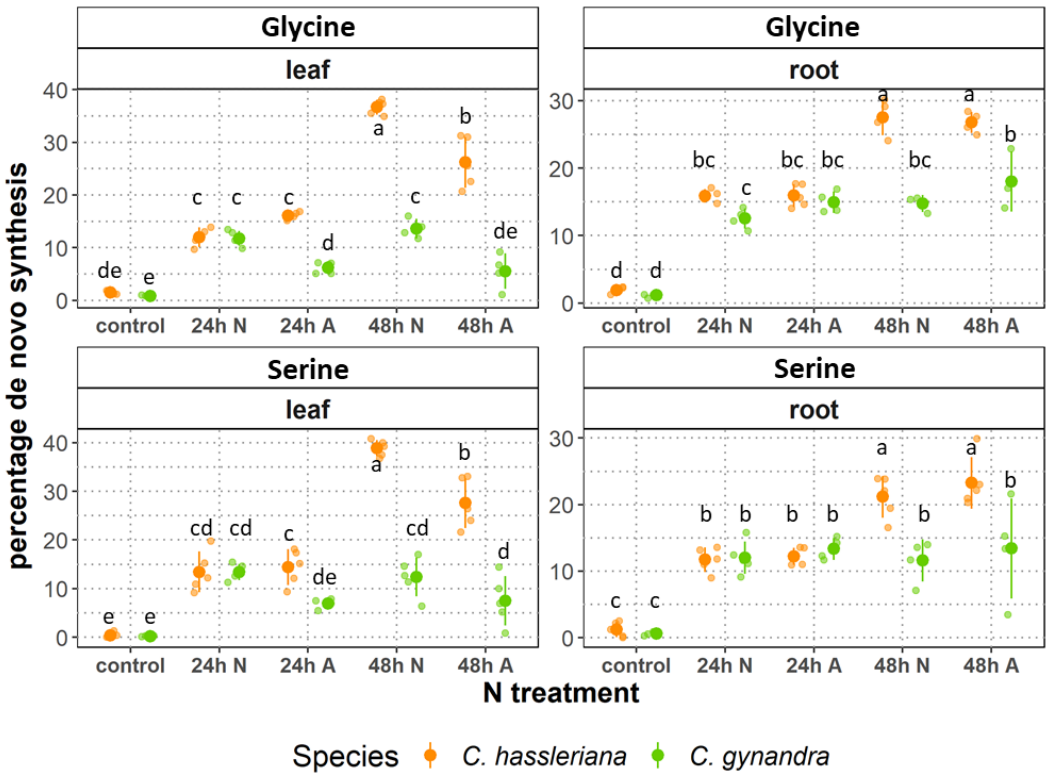

Figure 39: Percentage of *de novo* synthesized glutamate and proline in leaves and roots of *C. hassleriana* (C_3) and *C. gynandra* (C_4) after re-supply with nitrate or ammonium. Plants were bottom watered with 1 mM Hoagland solution for 2 weeks before being transferred to $^{15}NO_3^-$ and $^{15}NH_4^+$ nutrient solution. respectively. Samples for measuring incorporation of ^{15}N into amino acids were taken 24 h and 48 h after transfer to either nutrient solution. The letters A and N after the timepoints indicate ammonium and nitrate nutrient solution, respectively. (n=4)

As for Glu and Pro, *de novo* synthesis of both Ser and Gly was comparable in the 24 h N treatment but was relatively reduced in the A treatment in leaves of the C_4 species. These two amino acids also only showed further incorporation of the ^{15}N-label in the C_3 species. After 48 h the C_3 species showed a relative reduction in *de novo* synthesis in the A treatment compared to N-treated plants only in leaves, a similar pattern to that of Pro.

In C_4 species of the NAD-malic enzyme subtype, such as *C. gynandra*, alanine (Ala) and aspartate (Asp) are essential transfer metabolites. Therefore, the expression and activity of alanine and aspartate

aminotransferases (AT) are increased in leaves. The pyruvate-derived amino acid Ala showed a similar pattern as described for Pro, Gly and Ser (Figure 40).

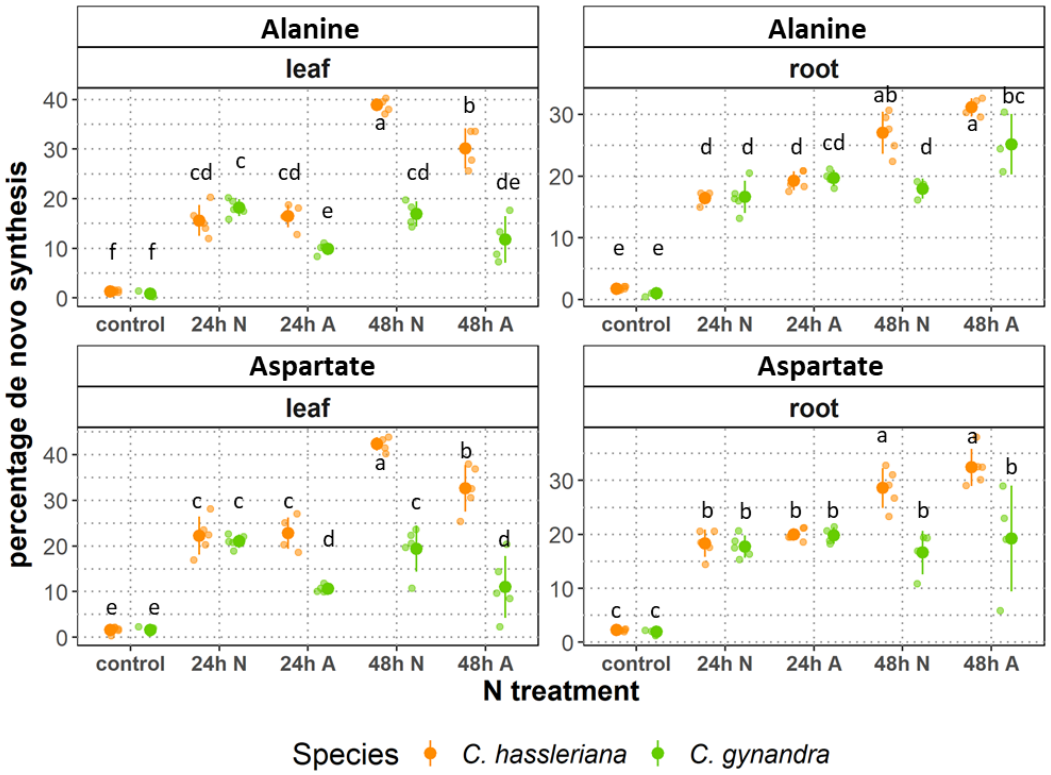

Figure 40: **Percentage of *de novo* synthesized alanine and aspartate in leaves and roots of *C. hassleriana* (C_3) and *C. gynandra* (C_4) after re-supply with nitrate or ammonium.** Plants were bottom watered with 1 mM Hoagland solution for 2 weeks before being transferred to $^{15}NO_3^-$ and $^{15}NH_4^+$ nutrient solution. respectively. Samples for measuring incorporation of ^{15}N into amino acids were taken 24 h and 48 h after transfer to either nutrient solution. The letters A and N after the timepoints indicate ammonium and nitrate nutrient solution, respectively. (n=4)

The only exception is a further increase in de novo synthesized Ala in 48 h A treatment in the C_4 species. Asp was synthesized at comparable rates after 24 h in the N and A treatment in the roots of both species but showed a decreased production rate in the A treatment in the leaves of the C_4 species (Figure 40).

5. Discussion

The CO_2 concentrating mechanism of C_4 plants not only facilitates more efficient photosynthesis, but also increases both the nitrogen and water use efficiency compared to C_3 species, especially under optimal N supply (Ghannoum *et al.*, 2011). Comparative studies between C_3 and C_4 species identified a strong positive correlation between leaf nitrogen content and the rate of photosynthesis in light-saturated conditions (Ghannoum *et al.*, 2011). Photosynthesis was shown to stimulate N uptake and assimilation through light-induced signalling cascades regulating the expression of NR in spinach, maize, rice and lettuce (Huber *et al.*, 1992, 1994; Ali, Sivakami and Raghuram, 2007; Lillo, 2008; Zhou, Liu and Yang, 2012). This is consistent with N metabolism being tightly linked to photosynthesis due to their co-localization in the chloroplast and the provision of reducing equivalents for N assimilation by the photosynthetic electron transport. C_4 species exhibit an improved photosynthetic nitrogen use efficiency (PNUE) also under low N conditions, implying that they might be less sensitive to N deficiency conditions, i.e., that they have a higher N deficiency tolerance than their C_3 relatives and potentially C_3-C_4 intermediate species. To determine whether the C_4 species *C. gynandra* has a higher N deficiency tolerance compared to its C_3 relative *C. hassleriana* and whether the C_3-C_4 intermediate species from the *Brassicaceae* family are more tolerant than their C_3 relatives, responses of these species to N deficiency were analysed.

5.1. Nitrogen deficiency responses vary between closely related *Brassicales* species with different photosynthesis types

5.1.1. Photosynthetic efficiency and biomass of C_4 *Cleome* species are less affected by N deficiency than in closely related C_3 and C_3C_4 intermediate species

Analyses of changes in biomass in C_3 and C_4 *Cleome* species grown in different nitrate conditions revealed differences in their responses to reduced nitrate availability. The Root/Shoot-Ratio of both species increased with decreasing nitrate concentration in the medium. This increase was due to increased root growth, which is a common response to low N availability (Gruber *et al.*, 2013). In contrast, the significant reduction of total fresh weight in the C_3- but not the C_4 species at low nitrate conditions (1 mM, 0.5 mM) indicates that the C_4 species is overall less affected by N deficiency. However, this improved tolerance to reduced nitrate availability was not observed in any of the C_3C_4-intermediate species of the *Moricandia* and *Diplotaxis* genera. This could imply that improved tolerance to low N either requires full C_4 photosynthesis or that N deficiency responses vary between genera.

This difference in N deficiency tolerance between C_3, C_3C_4-intermediate, and C_4 species was also reflected in their photosynthesis rates. Maximal carbon assimilation was reduced, and CO_2 compensation points were increased in the C_3 but not the C_4 *Cleome* species under 0.5 mM N nutrient solution. In C_3 species, the initial slope, referred to as mesophyll conductance, is proportional to the maximum activity of RuBisCO and therefore reflects carboxylation efficiency (Farquhar, Caemmerer and Berry, 1980). The increase of the CO_2 compensation point resulting from a decrease in the mesophyll conductance (initial slope, s. Supplemental Figure 1) in the C_3 species in the 0.5 mM N treatment points to a reduction in carboxylation efficiency under N deficiency. C_3 species invest a higher percentage of their leaf N content into production of RuBisCO (~15-30 %) than C_4 species (~5-10 %) (Evans, 1983; Evans and Seemann, 1984; Sage, Pearcy and Seemann, 1987; Terashima and Evans, 1988; Makino and Osmond, 1991; Poorter and Evans, 1998; Makino *et al.*, 2003; Ghannoum *et al.*, 2005; Tazoe, Noguchi and Terashima, 2006). During N deficiency the RuBisCO levels and activity might be reduced due to a lack of N, leading to the observed decrease in CO_2 assimilation rates. In contrast, in C_4 species, mesophyll conductance is dependent on the carboxylation efficiency of PEPC, however, at higher CO_2 concentrations, the maximal CO_2 assimilation rate is determined by the RuBP regeneration rate (Collatz, Ribas-Carbo and Berry, 1992; Bowyer and Leegood, 1997). Neither the initial slope nor the maximal assimilation rate was significantly reduced under low N conditions in the C_4 species, pointing to these processes not being limited by N deficiency. This is consistent with previous studies in maize which showed that N deficiency only resulted in a decrease in the maximum CO_2 assimilation rate in light-saturated conditions (Khamis and Lamaze, 1990; Champigny, 1995). On the contrary, a slight increase of the initial slope in the low N treatments could indicate an induction of PEPC expression or activity during N deficiency. This is consistent with an accumulation of PEPC protein observed in leaves of N-deficient maize plants after supply of nitrate or glutamine (Sugiharto *et al.*, 1990, 1992; Rajagopalan, Devi and Raghavendra, 1994). Similarly, nitrate was proposed to function as a signal metabolite modulating the activity of PEPC by activating the cytosolic protein kinase in wheat (Champigny and Foyer, 1992). Unlike their C_4 counterparts, which play a crucial role in CO_2 assimilation, C_3 PEPC isoforms do not exhibit any photosynthetic function and differ in enzymatic properties. C_4 PEPCs exhibit higher and lower K_m values for PEP and bicarbonate, respectively, and are more tolerant to feedback inhibition by malate than the C_3 isoforms (Ting and Osmond, 1973; Bauwe and Chollet, 1986; Dong *et al.*, 1998; Bläsing *et al.*, 2002; Westhoff and Gowik, 2004). In transgenic rice plants expressing the sugar cane (C_4) PEPC gene (tPEPC) TCA cycle and glycolysis genes showed different expression patterns than in the WT under different nitrogen concentrations, demonstrating an additional function of PEPCs expression in C_4 species, which differentially regulates N metabolism depending on N availability (Lian *et al.*, 2021). Comparative studies investigating the evolution of the PEPC protein in the *Flaveria* genus revealed that enzymatic properties of PEPCs from C_3C_4 intermediate

species are more similar to C_3 than to C_4 isoforms but show a higher expression level of PEPC typical for C_4 species (Westhoff and Gowik, 2004).

Carbon assimilation rates of the C_3 and the two C_3C_4 intermediate *Moricandia* species were significantly reduced in response to N deficiency conditions. This reduction in photosynthetic efficiency points to C_3C_4 intermediates lacking the improved N deficiency tolerance observed in the C_4 species. In accordance, multiple studies investigating the nitrogen use efficiency of C_3C_4 intermediate species in various genera, including monocots and dicots, also reported no differences in NUE of C_3 and C_3C_4 intermediate species (Monson, 1989; Pinto, Tissue and Ghannoum, 2011; Vogan and Sage, 2011). However, one of the intermediate species, *M. arvensis*, showed higher maximal assimilation rates than both other *Moricandia* species under both normal and low N conditions. A comparative analysis of transcript levels of various C_4-related genes in C_3C_4 intermediate *Moricandia* species found two *PEPC* transcripts with C_3-like characteristics expressed in leaves, one of which was less expressed compared to the C_3 species while expression of the lower-abundant isoform was increased (Paulus, Schlieper and Groth, 2013; Schlüter *et al.*, 2017). Their transcriptome analysis, however, could not identify a clear C_3C_4-related transcript pattern indicating a higher degree of species-specific differences compared to similar studies in C_3 and C_4 species which might explain the observed differences in CO_2 assimilation rates between *M. suffruticosa* and *M. arvensis* (Bräutigam *et al.*, 2011, 2014; Gowik *et al.*, 2011; Schlüter *et al.*, 2017). Overall, this indicates that the C-N metabolism of *Moricandia* intermediates is more like that of a C_3 rather than a C_4 species and points to the improved N deficiency tolerance being dependent on a complete transition to C_4 photosynthesis.

5.1.2. Higher accumulation of N in leaves might contribute to higher N deficiency tolerance of C_4 *Cleome* species

The difference in N deficiency tolerance was further investigated by analysing anion content, revealing differences in nutrient distribution between roots and shoots. In the C_4 species, accumulation of both NO_3^- and PO_4^{3-} in shoots was negatively correlated to the nitrate concentration in the nutrient solution (mNO_3^-), implying an increased allocation of these nutrients to the shoots in response to low nitrate conditions. Accumulation of PO_4^{3-} under low N conditions was also observed in maize (Schlüter *et al.*, 2012). This differential allocation of NO_3^- between roots to shoots was not observed in the C_3 or C_3C_4 intermediate species and could indicate a decrease in NO_3^- assimilation in shoots in the C_4 species. Analysis of natural variation in Arabidopsis regarding the adaptation to low N conditions suggests that the ability to accumulate and keep nitrate reserves under N deficiency conditions is correlated with the ability to downregulate nitrate reduction rates (North *et al.*, 2009). Consistent with this, pakchoi

(*Brassica Campestris* L.ssp. Chinensis(L.)) genotypes accumulating higher levels of nitrate exhibited lower expression of nitrate reductase genes than genotypes with low nitrate content (Luo *et al.*, 2006). In contrast, a similar study in spinach (*Spinacia oleracea L.*) found differences in NO_3^- uptake but not NR activity between varieties, suggesting that a higher NO_3^- uptake and transport capacity exceeding its reduction rates may be responsible for higher NO_3^- accumulation (X. Wang *et al.*, 2018). Uptake experiments performed in the C_3 and C_4 *Cleome* species confirmed a higher NO_3^- uptake and transport ability of the C_4 species (s. 4.3.2). A higher expression level of the NR encoding genes *NIA1* and *NIA2* in shoots of the C_4 species under low N conditions (s. 4.3.3), however, suggests that the mechanism underlying the NO_3^- accumulation in the C_4 species differs from both described strategies. Nevertheless, this ability of C_4 species to accumulate and keep nitrate reserves under N deficiency conditions not present in the C_3 or C_3C_4 intermediate species might contribute to its higher N deficiency tolerance. While this could hint at this adaptation to low N conditions being related to C_4 photosynthesis, the metabolic connection between both findings remains unclear.

C_4 plants have been shown to have a higher photosynthetic nitrogen use efficiency (PNUE), meaning they can accumulate more biomass than C_3 plants using less leaf nitrogen and RuBisCO (Bolton and Brown, 1980; Schmitt and Edwards, 1981; Sage, Pearcy and Seemann, 1987; Makino *et al.*, 2003). The EA IRMS analysis revealed that while overall N content was comparable in both *Cleome* species, significantly more N was found in the shoots of the C_4 species. This is surprising, since comparative studies in C_3 and C_4 grass species determined that C_3 and C_4 species have similar leaf N concentrations relative to their dry mass (Ghannoum *et al.*, 2005, 2011). This discrepancy could be explained by *C. gynandra* belonging to the NAD-ME subtype, possessing a higher N requirement than NADP-ME due to its lower photosynthetic nitrogen use efficiencies (PNUE). Thus, they invest a higher percentage of leaf N content in soluble protein and RuBisCO relative to NADP-ME species. This difference is a result of the higher k_{cat} of RuBisCO in the NADP-ME subtype (Ghannoum *et al.*, 2011). In the C_3 species, N content was already significantly reduced in mild N deficiency (1 mM N). In contrast, in *C. gynandra* total N levels were only significantly reduced in response to the lowest nitrate concentration (0.25 mM), which might be explained by the accumulation of NO_3^- reserves in the C_4 species. In general, C_4 species invest less N into soluble proteins, mainly RuBisCO, than C_3 species leading to a lower N consumption (Sage, Pearcy and Seemann, 1987; Evans and von Caemmerer, 2000).

5.1.3. Metabolic profiling reveals differential regulation of amino acid metabolism and the TCA cycle between C_3 and C_4 *Cleome* species in both normal and low N conditions

The mechanism underlying the improved N deficiency tolerance of the C_4 species observed in previous N deficiency experiments was further investigated using GCMS. Metabolite profiling performed on roots and shoots of C_3 and C_4 *Cleome* plants reveals clear metabolic differences in the accumulation of amino acids, TCA cycle intermediates and sugars in normal as well as low N conditions.

The metabolic profiling showed a higher accumulation of most amino acids in the C_4 species both under normal and low N conditions. This increased accumulation of free amino acids is in accordance with a comparative transcriptome analysis of a C_3 and C_4 *Cleome* species, in which an upregulation of N metabolism genes and lower steady state transcript levels of protein synthesis genes in the C_4 species were observed (Bräutigam *et al.*, 2011). Lower levels of soluble protein content detected in the leaves of the C_4 species support this finding. The C_4 species' lower RuBisCO demand might lead to a decrease in the synthesis of proteins for central carbon metabolism, thus overall increasing the photosynthetic NUE (Oaks, 1994). The accumulation of free amino acids in the C_4 species might act as a N reserve and confer the observed improved N deficiency tolerance, as parts of assimilated N have been shown to be stored in leaf vacuoles in the form of nitrate, amino acids and proteins to not limit growth during N deficiency. This is consistent with studies in rapeseed and soybean, which found that greater amino acid allocation and remobilization efficiency lead to higher NUE and yield under N deficiency (Liu *et al.*, 2020; Liang *et al.*, 2023).

The higher Ala and Asp content observed in the C_4 species, can be explained by their function as essential transfer metabolites in C_4 photosynthetic plants and is consistent with reports of increased transcript levels of AspATs and AlaATs in mature leaves of C_4 species, including *C. gynandra* (Schlüter *et al.*, 2019). AlaATs play a role in gluconeogenesis, glycolysis, amino acid metabolism, photorespiration, and C_4 photosynthesis (Hatch and Mau, 1977; Liepman and Olsen, 2001, 2003; Miyashita *et al.*, 2007). Tissue-specific over-expression of the barley homolog HvAlaAT has further been shown to improve NUE in Arabidopsis, rice and canola (Good *et al.*, 2007; Shrawat *et al.*, 2008; McAllister and Good, 2015). These observed alterations in NUE phenotype included increased root growth, larger leaf area and differences in partitioning of soluble sugars and amino acid uptake (McAllister and Good, 2015). Upregulation and differences in the expression patterns of AlaATs in C_4 species as described by Schlüter *et al.* could therefore cause alterations in overall N and C metabolism. These differences might then contribute to a better regulation of responses to changes in the C/N-balance in the C_4 species and a higher N deficiency tolerance. In line with this, Ala content was increased in the C_4 species but decreased in the C_3 species under N deficiency in our study. Metabolic

profiling in the NADP-ME C_4 species maize, however, showed a decrease of certain amino acids including Ala in leaves under N deficiency (Schlüter *et al.*, 2012), again hinting at differences in the N metabolism between NADP-ME and NAD-ME subtypes. AspAT transcript levels in C_4 species were higher than in the C_3 species, but the expression patterns of subcellularly localised isoforms varied between NADP-ME, NAD-ME subtypes, and amongst NAD-ME species (Schlüter *et al.*, 2019). Asp levels decreased significantly in the C_4 but not the C_3 species with decreasing N availability, correlating to the results of a study with transgenic rice plants expressing the PEPC gene from the C_4 species sugar cane (tPEPC). In this study the Asp levels showed opposite trends in WT and the tPEPC plants, with decreasing N supply with relatively higher Asp levels in tPEPC in normal N conditions but relatively lower levels under nitrogen deficiency (Lian *et al.*, 2021).

The accumulation pattern of Lys is unique among the amino acids in the C_4 species. Lys is an essential amino acid synthesized from Glu via the plastidial Asp-family pathway together with (Met), threonine (Thr) and isoleucine (Ile). The much stronger decrease of Lys levels in response to N deficiency conditions observed in the C_4 species could indicate the induction of Lys catabolism via a bifunctional polypeptide Lys–ketoglutarate reductase (LKR)/ saccharopine dehydrogenase (SDH) (Stepansky et al., 2006). In case of energy deficiency, the enzyme converts Lys back to Glu and subsequently Acetyl-CoA, thereby providing inputs for the TCA cycle. Similarly, the other Asp-derived amino acids can also either flux directly into the TCA cycle (Ile) or be converted to Ile (Met, Thr) (Stepansky *et al.*, 2006; Wenyi Wang *et al.*, 2018). Lys has further been shown to not only alter the accumulation of other amino acids in the aspartate family, but also those from other pathways, e.g., high lysine inducing accumulation of tryptophan (Trp) and Gly in transgenic rice (Yang *et al.*, 2016; Yang, Zhao and Liu, 2020). The connection between Lys and Gly content could also explain the observed decrease in Gly levels in low N conditions as a result of the strong decrease in Lys. Catabolism of Lys has been reported to play a role in the alteration of metabolic pathways possibly related to its role in seed development in monocots and dicots. The stress response and its connection with metabolism, however differed between Arabidopsis and maize, which could be attributed to a difference between dicots and monocots but might also be related to metabolic differences between C_3 and C_4 photosynthetic plants (Moulin, Deleu and Larher, 2000; Galili, 2002; Moulin *et al.*, 2006; Less *et al.*, 2011; Kiyota, Pena and Arruda, 2015).

Both Gly and Ser levels are significantly higher in the C_4 species in control conditions, which is in line with the overall higher amino acid content of the C_4 species. In response to N deficiency, Gly levels increased slightly in the C_3 and decreased in the C_4 species, respectively. The decrease in Gly levels in the C_4 species is most likely a result of the aforementioned strong decrease in Lys levels. Accumulation of Gly in the C_3 species could indicate a low energy level of the plant due to the low N conditions and a subsequent decrease in protein synthesis rates. Ser accumulated under low N conditions in both

Cleome species, albeit more strongly in the C_3 species. As part of photorespiration, one molecule of Ser is produced from two molecules of Gly by the GDC/SHMT-complex in the mitochondria. Due to the reduced oxygenation rate achieved through the CCM, rates of photorespiration are reduced in C_4 plants. The phosphorylated pathway of serine biosynthesis (PPSB) located in plastids, is one of two alternative pathways, by which the plant can produce Ser, the other one being the glycerate pathway (located in peroxisomes)(Rosa-Téllez *et al.*, 2024). Metabolic analyses of an Arabidopsis mutant with moderately impaired photorespiration (*hpr1*) revealed accumulation of the photorespiration intermediates Gly, Ser, glycerate, hydroxypyruvate and ethanolamine (Timm *et al.*, 2021). Except for hydroxypyruvate, which was not detected and Gly content, which was reduced, this matches the metabolic profile of the C_4 species under N deficiency conditions. Moreover, *hpr1* showed an increased accumulation of the S metabolites OAS, Cys and GSH, consistent with the increased accumulation of Cys and GSH observed in leaves of C_4 *Flaveria* species. This alteration in S metabolism was found to be controlled by the root and correlated to relocation of GSH metabolism to the root and the PPSB was proposed Ser biosynthesis to be the main source of Ser in C_4 species (Bräutigam and Gowik, 2016; Gerlich *et al.*, 2018b). However, more recent studies have demonstrated that the PPSB is also essential for C_3 plants as photorespiratory Ser was insufficient to compensate for PPSB-mediated Ser biosynthesis (Zimmermann et al., 2021). Studies on the interaction of the PPSB and GPSB conducted by Rosa-Téllez et al. suggest a complex interplay between both pathways affecting N, S, and C metabolism but also a high plasticity of the involved metabolic pathways (Rosa-Téllez *et al.*, 2024).

The regulation of amino acid metabolism is closely interconnected with the TCA cycle since NH_4^+ assimilation via the GS/GOGAT cycle, requires ATP, reductants (reduced NADH, ferredoxin) and C skeletons in the form of 2OG (Lea and Ireland, 1999). Under control conditions (4 mM N), TCA cycle intermediates were present in similar amounts in the C_3 and C_4 species. Due to the CCM, organic acids are usually more abundant in C_4 leaves than C_3 (Fan *et al.*, 2024). This was the case for malate, citrate and 2OG. However, a previously reported 2-fold higher level of malate, pyruvate and 2-oxoglutarate in a C_4 NADP-ME type species compared to a C_3 species was not observed in the *Cleome* species, which might be related to differences between NADP-ME and NAD-ME species (Arrivault *et al.*, 2017; Borghi *et al.*, 2022). Another exception from this is pyruvate which is more abundant in the C_3 species under control conditions. This is likely due to the importance of pyruvate for the regeneration of PEP in C_4 plants (Hatch, 1987; Ludwig, 2016). In line with this, pyruvate levels decreased more strongly in the C_3 species in response to N deficiency. The bigger pool of alanine in the C_4 species might allow for more robust pyruvate levels during N deficiency. Besides pyruvate, malate and fumarate, all TCA cycle intermediates accumulated in the C_3 species under N deficiency conditions. This is consistent with the TCA cycle being inhibited in leaves in low N conditions (Schlüter *et al.*, 2012; Li *et al.*, 2018; Sun *et al.*,

2021; Xue *et al.*, 2022). In contrast, in the C_4 species only malate, 2OG, and, to a smaller extent, succinate and isocitrate accumulated in response to N deficiency.

While their levels under control conditions were higher in the C_4 than the C_3 species, citrate and malate are the most abundant TCA cycle intermediates in both species, which could indicate the TCA cycle operating in an incomplete (open) mode (Igamberdiev and Eprintsev, 2016). The incomplete cycle is implemented mainly in photorespiratory conditions and consists of two branches producing malate and citrate, respectively (Igamberdiev *et al.*, 2001; Igamberdiev and Gardeström, 2003; Gardeström and Igamberdiev, 2016). In Arabidopsis, high citrate concentration lead to changes in the abundance of transcripts involved specifically in the TCA, nitrogen metabolism, sulfur metabolism, and DNA synthesis (Finkemeier *et al.*, 2013). High citrate accumulation is further associated with photorespiration in C_3 plants, as it supplies reduction power in the cytosol in form of NADH via isocitrate dehydrogenase and the conversion of 2-oxoglutarate to glutamate (Tcherkez *et al.*, 2009). However, no increase in glutamate levels was observed, while 2-oxoglutarate levels increased which could indicate that glutamate is quickly converted to glutamine either in the cytosol or chloroplast using ammonia, as an increase in glutamine levels was observed in C_3 leaves. The accumulation of malate levels in N deficiency conditions only in the C_4 species shows a differential regulation of the organic acid pool between C_3 and C_4 plants in the context of N deficiency.

Another intermediate accumulating in both species was 2OG, derived from sugar respiration or amino acid transamination reactions catalysed by various enzymes including isocitrate dehydrogenases, amino transaminases, and Glu dehydrogenases. GDH can catalyse both the biosynthetic amination reaction, producing Glu and the catabolic de-amination reaction which produces 2OG. In the presence of ammonium, Gln, or sugars Glu production is favoured, while nitrate, Glu, or C limitation favours the catabolic de-amination reaction (production of 2OG) (Lancien, Gadal and Hodges, 2002). Therefore, 2OG is a key metabolite in the coordination of carbon/nitrogen metabolism (Hodges, 2002) and has been shown to directly regulate the activities of the cytosolic pyruvate kinase, PEP carboxylase and the mitochondrial citrate synthase (Lancien, Gadal and Hodges, 2000; Ferrario-Méry *et al.*, 2001). These findings suggest that mitochondrial TCA cycle enzymes contribute substantially to the regulation of N assimilation and amino acid biosynthesis in leaves (Araújo *et al.*, 2014)

The synthesis of the nonproteinogenic amino acid GABA from Glu is catalysed by the enzyme Glu decarboxylase (GAD) (Zik *et al.*, 1998). It represents the first step of the so-called GABA shunt, a three-step pathway producing succinate from 2OG bypassing the TCA-cycle (Bouché *et al.*, 2004). The observed decrease in GABA levels and increase of succinate content under low N conditions is consistent with the activation of the GABA shunt. GABA has been previously proposed to play a role in the regulation and maintenance of the C/N balance (Fait *et al.*, 2011). More recent studies further

demonstrate that exogenous GABA application changes carbon and nitrogen fluxes in poplar seedlings under low nitrogen conditions by altering nitrate reductase, nitrite reductase and glutamine synthetase activities (Chen *et al.*, 2020). However, so far, no direct link between differences in GABA metabolism and C_3 or C_4 photosynthesis has been identified.

5.1.4. Secondary metabolites accumulate in response to low N condition only in C_3 species, whereas sugars accumulate in both C_3 and C_4 *Cleome* species

The metabolic profiling revealed the accumulation of various sugars, most significantly sucrose, in both *Cleome* species in roots and shoots during N deficiency. This observation complements previous studies that reported the accumulation of unstructured carbohydrates in tobacco leaves in response to low N conditions (Paul and Driscoll, 1997; Geiger *et al.*, 1999). Paponov *et al.* further showed that N deficiency can alter distribution of sugars between the different sink organs in maize, namely increasing partitioning towards roots and shoots. This is consistent with my findings and might indicate a role of N in sugar unloading (Paponov and Engels, 2005; Zhang *et al.*, 2021). The accumulation of carbohydrates might act as a key signal to decrease photosynthesis and TCA cycle activity in plant leaves during N deficiency (Schlüter *et al.*, 2012; Li *et al.*, 2018). Levels of glucose and sucrose were significantly higher in the leaves of the C_3 species throughout all N treatments. This correlates with findings by Barbehenn *et al.* which observed a higher accumulation of sucrose and other non-structural carbohydrates in C_3 compared to C_4 grasses (Barbehenn *et al.*, 2004) potentially contributing to the stronger reduction of photosynthesis rates in the C_3 species under N deficiency.

Accumulation of various secondary metabolites, including shikimate, glycerate, myoinositol, threonate, sitosterol and sinapinate under N deficiency conditions, was only observed in the C_3 species. Not all of these specific metabolites have been directly linked to N limitation, but most of them play a role in abiotic stress responses. Myo-inositol acts as a precursor for a number of lipid signalling molecules with diverse functions mainly in abiotic stress responses, but also in the regulation of cell death, auxin perception, and cell wall biosynthesis (Eckardt, 2010; Hou, Ufer and Bartels, 2016). Shikimate is not only the precursor for the biosynthesis of the aromatic amino acids (Trp, Tyr and Phe) but also plays an important role in secondary metabolism such as the production of various plant hormones, flavonoids, and phenylpropanoids e.g. sinapinate (Yan-li et al., 2023). Activation of the shikimate pathway is associated with abiotic stress conditions and leads to the accumulation of high levels of aromatic amino acids and related secondary metabolites (Maeda and Dudareva, 2012; Francini, Giro and Ferrante, 2019). The accumulation of shikimate and various related secondary metabolites under N deficiency conditions in the C_3 but not the C_4 species therefore implies that only

the C_3 species exhibits significant stress responses in the low N treatments, supporting the hypothesis of the C_4 species being more tolerant to N deficiency. One exception was ethanolamine, which only seemed to be affected by N deficiency in the C_4 species. Ethanolamine can be synthesized by direct decarboxylation of Ser catalysed by a pyridoxal 5'-phosphate-dependent l-serine decarboxylase (SDC) (Rontein et al., 2001). Moreover, studies in on N limitation in microorganisms found ethanolamine to be a possible alternative N source (Krysenko and Wohlleben, 2022).

Overall, significant differences in accumulation patterns of sugars, amino acids and TCA intermediates between the C_3 and C_4 species under N deficiency conditions suggest a differential regulation of N deficiency responses in C_3 and C_4 species. Increased production of secondary metabolites via the shikimate pathway under low N conditions was observed only in the C_3 species, indicating a higher stress level than in the C_4 species.

5.2. Ammonium sensitivity varies between closely related *Brassicales* species with different photosynthesis types

Plants can take up inorganic N in the form of NO_3^- and NH_4^+ (A. J. Miller and Cramer, 2005). Preference of one N source over the other is highly species-specific and might manifest in higher biomass production or increased accumulation of N and N-containing compounds such as amino acids. High levels of NH_4^+ are known to inhibit growth in many higher plants, especially many crops, e.g., potato, tomato etc. (Britto and Kronzucker, 2002). However, many abiotic factors such as pH, temperature and general nutrient availability affect the N source preference in any given environment (Britto and Kronzucker, 2013). Like NH_4^+, NO_3^- assimilation also has its drawbacks as the reduction of NO_3^- to NH_4^+ is an energetically costly process and therefore depends on light for energy supply. NO_3^- fertilizers are frequently used in agriculture but only 30-40 % of applied N can be used by the plants while the rest is lost and contributes to environmental problems like water pollution (Raun and Johnson, 1999; Directorate-General, 2002; Kant, Bi and Rothstein, 2011). Furthermore, the speciation of N in the soil is strongly affected by a bacterial interconversion between NH_4^+ and NO_3^-. Due to bacterial oxidation of NH_4^+ to NO_3^- (nitrification), NO_3^- is the most abundant N source for plants (Liang and MacKenzie, 1994; Kaboneka, Sabbe and Mauromoustakos, 1997). However, in acidic or waterlogged soils nitrification is reduced, dramatically altering the N sources available to plants. These issues highlight the importance of a better understanding of the molecular mechanisms underlying the assimilation of NO_3^- and NH_4^+ as well as their interplay to reduce the need for fertilizers by improving NUE.

5.2.1. Supply of additional ammonium offsets reduced biomass caused by N deficiency in C_3 but not C_4 *Cleome* species

Growing C_3 and C_4 *Cleome* species in various compositions of NO_3^- and NH_4^+ showed that neither *Cleome* species is dependent on a specific N source as no significant changes in biomass were observed. On the contrary, both species grew best when provided with a mixture of both NO_3^- and NH_4^+. Supply of both N sources has been shown to result in optimal growth many plant species (Haynes and Goh, 1978; A J Miller and Cramer, 2005). This was further confirmed by a study modelling the energy costs of amino acid biosynthesis in photoautotrophic and heterotrophic growth conditions in Arabidopsis, which found combined uptake of both N sources allowed for the most efficient utilization of energy sources (Arnold, Sajitz-Hermstein and Nikoloski, 2015). There were, however, differences in the optimal amount of NH_4^+, as *C. gynandra* seemed to prefer lower NH_4^+ concentrations (10 to 25 %) than *C. hassleriana* (25 to 50 %) for optimal growth. Toxic effects of NH_4^+ are caused by an imbalance of NH_4^+ absorption, production, and consumption (Shilpha, Song and Jeong, 2023). These findings therefore imply that the relatively lower NH_4^+ tolerance of the C_4 species is a result of metabolic changes affecting either NH_4^+ production or assimilation. Reduced photorespiratory flux in the C_4 species lowers the amount of NH_4^+, which is released through the conversion of Gly to Ser. Therefore, the C_4 species might be less adapted to dealing with high NH_4^+ levels (Martin *et al.*, 1983). Furthermore, the localization of enzymes catalysing the assimilation of NH_4^+ and NO_3^-, GS1 and NR, in BS and M cells, respectively in *C. gynandra* restricts NH_4^+ assimilation to BS cells and might necessitate NH_4^+ transport. This might lower the rate of NH_4^+ assimilation in the C_4 *Cleome* species which could explain the relatively stronger reduction of biomass in response to the NH_4^+ treatments. Contrary to expectations, biomass of all *Moricandia* and *Diplotaxis* species, excluding *D. tenuifolia*, decreased in high NH_4^+ concentrations. The clear differences between the four C_3C_4 intermediate species indicate that they might use different strategies to deal with excess NH_4^+. Recent studies suggest that the photorespiratory pathway can operate in a non-cyclic manner allowing it to interact with other metabolic pathways, potentially offering more flexibility to adapt to environmental conditions. This could explain metabolic differences between different C_3C_4 intermediate species such as this difference in NH_4^+ tolerance (Timm *et al.*, 2012; Hodges *et al.*, 2016; Busch, 2020). Alternatively, rather than being related to photosynthesis type, this seemingly higher sensitivity to NH_4^+ might be species-specific as NH_4^+ tolerance can vary greatly between plant species, especially between domesticated and undomesticated plants (Britto and Kronzucker, 2002). This, however, seems less likely, since the biomass of two C_3 species *M. moricandioides* and *D. viminea*, similar to *C. hasslerina*, increased in the 10 % and 25 % treatments. While all tested *Brassicales* species were able to use NH_4^+ as their sole N source, it is still unclear whether NH_4^+ and NO_3^- can be used interchangeably. In this experiment, both

Cleome species showed a small reduction in biomass when grown with NH_4^+ as their only N source. Like the C_4 species, the two C_3C_4-intermediate *Diplotaxis* species only seemed to benefit from NH_4^+ in the absence of NO_3^-, while of the *Moricandia* intermediates only *M. suffruticosa* matched this pattern. Neither of the C_3 species from the *Moricandia* and *Diplotaxis* genus showed the beneficial effect of NH_4^+ on biomass observed in *C. hassleriana.*

Analysis of anion content in correlation to the N concentration in the nutrient solution (mNO_3^-) revealed an unchanged positive correlation to the NO_3^- content in roots, indicating that in C_4 species the uptake of NO_3^- is not affected by the presence of NH_4^+, also indicating that NO_3^- is the preferred N source of the C_4 species. In the C_3 species, the positive correlation between mNO_3^- and shoot NO_3^- content increased when NH_4^+ was present in the nutrient solution, implying an improved uptake of NO_3^-. In rice, a similar synergistic effect of combined NO_3^- and NH_4^+ on N acquisition was observed (Kronzucker *et al.*, 1999). The strong negative correlation between mNO_3^- and PO_4^{3-} as well as SO_4^{2-} levels in shoots indicates that accumulation of PO_4^{3-} and SO_4^{2-} is induced by NH_4^+. The addition of ammonia to the nutrient solution has been shown to significantly increase APR activity and flux through the sulfate assimilation pathway in shoots of Lemna and Arabidopsis (Brunold and Suter, 1984; Suter *et al.*, 1986; Koprivova *et al.*, 2000). However, unlike in the C_4 species, PO_4^{3-} and SO_4^{2-} content in C_3 seemed to be unaffected by NH_4^+. In C_3C_4 intermediate species, NO_3^- content in both roots and shoots showed a weaker positive correlation to mNO_3^-. This reduced correlation could indicate that C_3C_4 species depend less on the availability of NO_3^- which might be due to them being able to use NH_4^+ equally as a N source. The increased leaf Cl^- levels in all *Moricandia* and *Diplotaxis* species seems to be somewhat proportional to the increasing NH_4^+ in the medium and might therefore be caused by combined uptake of Cl^- and NH_4^+ since NH_4^+ is provided to the plants in the form of NH_4Cl. Recently, high Cl^- accumulation has been reported to be beneficial for tissue water balance photosynthesis performance, and water-use efficiency (Franco-Navarro *et al.*, 2016; Franco-Navarro *et al.*, 2019; Nieves-Cordones *et al.*, 2019). Rosales *et al.* further showed that Cl^- improves nitrate utilization by competitive exclusion of NO_3^- from the vacuoles in leaves (Wege, Gilliham and Henderson, 2017; Rosales *et al.*, 2020). This improved NUE due to the higher Cl^- concentration in the growth medium could also contribute to the comparably low biomass reduction in response to the NH_4^+ treatments.

5.2.2. Photosynthetic efficiency of C_3 and C_4 *Cleome* species is differently affected in the presence of ammonium

The combined effect of both N sources in relation to photosynthesis type was investigated through comparing their effect on photosynthetic efficiency of C_3 and C_4 *Cleome* plants. Compared to the previous experiment measuring CO_2 assimilation rates in the *Cleome* species in response to different NO_3^- concentrations, the maximal assimilation rates of both species were increased in this experiment. Furthermore, the assimilation rate of the C_4 species showed a stronger decrease in the N deficiency treatments than in the previous experiment. The photosynthesis data for the N deficiency experiment was acquired in December 2022 while the experiment investigating the NH_4^+ tolerance was performed in July 2023. The experiment was performed in a greenhouse, thus the changing light intensity outside due to seasonal changes might have affected the CO_2 assimilation in the C_4 species. This would indicate that the photosynthetic efficiency of the C_4 plants in the N deficiency experiment might have been limited by light. Mirroring the biomass results the C_3 species achieved the highest maximal CO_2 assimilation rates in treatments with a combination of both NH_4^+ and NO_3^- while in the C_4 species the CO_2 assimilation rate was highest in the NO_3^- treatment. This confirms that the increase in biomass of C_3 plants treated with NH_4^+ is a result of improved photosynthetic efficiency. Recent studies have shown that sufficient or elevated CO_2 conditions improve the CO_2 assimilation rate in C_3 species but not C_4 species when using NH_4^+ rather than NO_3^- as a source of N (Wang, Gao, Yong, Wang, *et al.*, 2020). NH_4^+ taken up from the soil is preferentially assimilated into organic compounds in the roots instead of being transported to the shoot (Xu, Fan and Miller, 2012).

5.3. N uptake and assimilation in C_3 and C_4 *Cleome* species

The contribution of differences in the uptake and assimilation of NO_3^- and NH_4^+ to their differential effects on C_3 and C_4 plants was investigated through re-supply experiments. They revealed a faster response of the C_4 species to newly available NO_3^-or a higher NO_3^- uptake ability. A decrease in NO_3^- content after 24 h in the C_4 species could indicate more efficient assimilation of NO_3^- into amino acids and proteins, an increase in soluble protein content was observed in roots 1 h after the re-supply. However, after 4 h protein levels were reduced back to control conditions (1 mM NO_3^-) and did not change again within the 24 h timeframe, indicating that protein turnover is likely not the reason for the decreased NO_3^- content after 24 h. In this assessment, fresh weight was used as the reference, which does not take subcellular localization and compartmentalization into account, thus neglecting the possibility of diluting the protein concentration by growth effects (Genard, Baldazzi and Gibon, 2014). Metabolic profiling found higher levels of free amino acids in C_4 species under both normal und

low N conditions (s. 4.1.4), which would be consistent with assimilation of the newly provided NO_3^- into amino acids within 24 h and their subsequent transfer to the shoots, possibly to act as a N reserve.

C_3 plants transferred to NH_4^+ nutrient solution did not show any significant changes in NO_3^- levels as expected. The increase of NO_3^- after 24 h in roots of C_4 plants, could be caused by bacterial oxidation, by which NH_4^+ from the nutrient solution is converted into NO_3^- and taken up by the plant. This hypothesis was tested by eliminating potential bacterial contamination through adding ampicillin to the nutrient solution, which did not alter the results. Thus, the increase in NO_3^- content in the C_4 species after the transfer to NH_4^+ solution is likely caused by small amounts of the N deficiency nutrient solution (1 mM NO_3^-) remaining in the vermiculite in the pot after the transfer. This uptake of even minuscule amounts of NO_3^- was not observed in the C_3 species grown in the same experimental setup, which could again hint at improved NO_3^- uptake in the C_4 compared to the C_3 species.

Quantification of uptake of ^{15}N-labeled potassium nitrate (KNO_3) and ammonium chloride (NH_4Cl) by N-deficient C_3 and C_4 *Cleome* plants showed a rapid uptake of N in roots when transferred to either N source, once again confirming that both species can utilize both N sources. Furthermore, the higher levels of N in the roots of the C_4 species demonstrate a higher NO_3^- uptake ability of the C_4 species. These findings suggest that there might be a difference in either expression or activity of nitrate transporters between the C_3 and C_4 species. Gene expression analysis revealed a significant increase of the *NRT1.1* expression under N deficiency conditions (1 mM N) in the C_4 species, while the gene was barely expressed in the C_3. While NRT1.1 is typically characterized as a dual-affinity nitrate transporter, some evidence points to it having a key role under low N conditions and acting like a nitrate sensor rather than a nitrate transporter (Huang *et al.*, 1996; Ye, Tian and Jin, 2019). Based on this, the superior N deficiency tolerance of the C_4 species might be at least partially due to improved nitrogen sensing under low N conditions. In a recent study, Wu *et al.* identified a TF (ZmEREB97), functioning as a key regulator of nitrate uptake in the C_4 species maize by directly targeting and inducing the expression of 6 *ZmNRT* genes (*ZmNRT1.1A*, *ZmNRT1.1B, ZmNRT1.2*, *ZmNRT2.1*, *ZmNRT2.5* and *ZmNRT3.1A*) by binding to the GCC-box motif in their promoters. *ZmEREB97* mRNA and protein was shown to accumulate in roots within 5 min upon nitrate supply. In *zmereb97* mutants, accumulation of biomass was impaired in N deficiency and full N conditions (Wu *et al.*, 2024). So far, it is still unknown whether this regulatory network is conserved amongst other species. Moreover, the expression of *NRT1.1* in the C_4 species further increased in the combined NH_4^+ and NO_3^- treatment suggesting an induction of *NRT1.1* expression by NH_4^+. In Arabidopsis, NRT1.1 has been shown to alleviate NH_4^+ toxicity in a NO_3^--dependant manner (Hachiya and Noguchi, 2011; Hachiya *et al.*, 2011), consistent with no increase in *NRT1.1* expression being observed in the NH_4^+-only treatment. The lack of an induction of *NRT1.1* in the C_3 species in the NH_4^+-treatments could indicate that the C_3 species does not perceive these NH_4^+

levels as toxicity, potentially due to its higher internal NH_4^+ levels. However, NH_4^+ taken up from the soil is mostly directly assimilated into amino acids in root cells and thus might not be related to photorespiratory NH_4^+ pools (Masclaux-Daubresse *et al.*, 2010). In wheat, higher NH_4^+ tolerance was correlated to enhanced transcriptional regulation of a vacuolar glucose transporter and glucose metabolism, which provided additional C skeletons in the form of particularly of 2-oxoglutarate and pyruvate under NH_4^+ stress (Hu *et al.*, 2024). Furthermore, increased glutamate levels under NH_4^+ conditions were shown to decrease levels of TCA cycle intermediates and ATP through inhibition of pyruvate kinase (PK) activity. This disruption of the TCA cycle then leads to reduced plant growth (Wang, Gao, Yong, Liu, *et al.*, 2020). The PK catalyzes conversion of PEP and ADP into pyruvate, generating ATP. However, due to the fundamental role of PEP in primary C fixation PK activity might generally be inhibited in C_4 plants, which could explain their higher NH_4^+ sensitivity. In addition, the ^{15}N uptake experiment showed a relative increase and decrease in N uptake, in the roots of the C_3 and C_4 species, respectively, when transferred to the NH_4^+-containing nutrient solution. This finding is in line with the C_3 species being able to benefit more from NH_4^+ supplementation. Interestingly, these higher N levels in the C_3 roots under NH_4^+ supply did not seem to translate to the shoots, which is consistent with a study in barley that showed differences in uptake and assimilation of NH_4^+ and NO_3^-. While NO_3 was predominantly transported to and assimilated in the shoot, transport of NH_4^+ to the shoot was much lower, suggesting NH_4^+ might be assimilated in the roots (Lewis and Chadwick, 1983). The uptake experiment further demonstrated that, while to lesser degree, the C_4 species can also take up N in the form NH_4^+, but the transport to the shoots is similarly low as in the C_3 species. This matches the results of a previous studies in maize, which found root to shoot transport of NH_4^+ to be insignificant compared to NO_3^- (Murphy and Lewisf, 1987).

This difference in NH_4^+ uptake, as in the case of NO_3^- uptake, could also be explained by differences in the expression or activity of ammonium transporters. The expression pattern of the high-affinity ammonium transporter AMT2.1 in multiple organs, including roots, shoots and stems, is conserved across various species including Arabidopsis, maize, sorghum, and sugarcane which could imply functional conservation. Highest expression of *AMT2.1* was generally observed in roots, where it was shown to play a role in ammonium uptake under N deficiency conditions (Sohlenkamp *et al.*, 2002; Koegel *et al.*, 2013; Giehl *et al.*, 2017; Dechorgnat *et al.*, 2019; Koltun *et al.*, 2022). However, in high N concentrations, AMT2.1 is mainly involved in xylem loading and root-to-shoot transport of NH_4^+ (Giehl *et al.*, 2017). In our experiments, in the C_3 species, *AMT2.1* expression was highest in the combined treatment, therefore coinciding with the optimum for photosynthetic efficiency and growth. Expression analysis further showed that the expression levels of the ammonium transporter *AMT2.1* were generally higher in the C_3 than in the C_4 species. This implies that both NH_4^+ uptake and root-shoot translocation of AMT2.1 are inhibited in the C_4 species which fits the lower NH_4^+ uptake rates

observed in the ^{15}N uptake experiment. Furthermore, AMT2.1 might also play a role in the recycling of photorespiratory NH_4^+ as transcripts levels have been shown to slightly decrease in response to elevated CO_2 levels (Sohlenkamp *et al.*, 2002). Owing to CO_2 concentrating mechanism of the C_4 plants it is possible that higher internal CO_2 concentration might interfere with optimal translocation of NH_4^+ to the shoots. Expression analysis of the two *NIA2* isoforms showed opposing expression patterns of both isoforms between the C_3 and the C_4 species. In the C_3 species, the NIA2 seems to be regulated by NO_3^- concentration as root and shoot expression of both isoforms was highest under full N conditions and strongly reduced in all other conditions, however NR activity did not change significantly. In contrast in the C_4 species, both isoforms were higher expressed in leaves and induced by low NO_3^- concentrations (1 mM), but their transcript levels varied depending on the presence of NH_4^+ rather than NO_3^-. However, changes of nitrate reductase activity in response to the different N treatments did not reflect this expression pattern. This is consistent with various studies in different species not detecting any direct effects of NO_3^- on NR activity, finding that NR activity was instead reduced by nitric oxide (NO) (Kaiser *et al.*, 2002; Du *et al.*, 2008; Rosales *et al.*, 2011). In addition to the reduction of nitrate, the NR enzyme can also catalyse reduction of nitrite to NO, a reaction inhibited in the presence of NO_3^- (Yamasaki, Sakihama and Takahashi, 1999; Rockel *et al.*, 2002). This could explain the strong, seemingly dose-dependent increases in NR activity observed in the C_4 roots in treatments containing NH_4^+. The increase in NR activity might be caused by the reduction of the inhibition by NO_3^- rather than an induction by the increasing NH_4^+ concentration. In rice, NO produced by NR was reported to improve N-use efficiency by increasing lateral root initiation and inorganic N uptake (Sun *et al.*, 2015). Moreover, studies in Arabidopsis demonstrated that NO is necessary for nitrate sensing in the soil (Nejamkin *et al.*, 2023). Overall, the expression analysis showed clear differences in the regulation of N uptake and assimilation between the C_3 and C_4 *Cleome* species. However, more studies are necessary to understand how these differences are connected to the photosynthesis mechanism.

To investigate the mechanism of NO_3^- and NH_4^+ assimilation and potential differences based on photosynthesis type, incorporation of $^{15}NO_3^-$ and $^{15}NH_4^+$ into proteins was measured in N-deficient C_3 and C_4 plants. The isotopologue profiling revealed significant differences in the amount of *de novo* synthesized amino acids between the C_3 and C_4 species, depending on the N source. The overall lower percentage of newly synthesized of amino acids observed in C_4 leaves indicates that the C_4 species has a lower demand for amino acids biosynthesis after N deficiency. This finding is consistent with higher amount of free amino acids in the C_4 species and reinforces their hypothesized role as a N storage. The *de novo* synthesis rates of most amino acids plateaued after 24 h in the C_4 species irrespective of N source. In contrast, further increases were observed after 48 h in the C_3 species. This indicates that the C_3 species overall has a higher protein turn-over rate compared to the C_4 species. The need for a higher

protein synthesis rate is likely related to the higher protein-to-fresh weight ratio and higher RuBisCO content in C_3 leaves compared to C_4 leaves (Ku, Schmitt and Edwards, 1979; Bräutigam *et al.*, 2011). A comparative transcriptome analysis of C_3 and C_4 *Cleome* species further revealed that C_4 species not only had lower steady-state mRNA levels of CBBC and photorespiration genes but also of genes involved in protein synthesis and the amino acid metabolism (Bräutigam *et al.*, 2011). The reduced production of proteins in the leaves of C_4 plants likely contributes to their improved nitrogen use efficiency (Oaks, 1994). The relative reduction in *de novo* synthesis compared to the NO_3^- treatment in leaves could be due to differences in N leaf metabolism as a consequence of C_4 photosynthetic evolution. Higher photorespiration rates in the C_3 species and subsequent re-assimilation of the photorespiratory NH_4^+ might explain the higher turnover of amino acids observed in the C_3 species. In the C_4 species, the NH_4^+ treatment had contrasting effects on leaves and roots. The accelerated *de novo* synthesis of most amino acids in the roots in the NH_4^+ treatment is consistent with the additional NH_4^+ being assimilated into amino acids in the roots (Masclaux-Daubresse *et al.*, 2010). Alternatively, the reduction in the leaves might be caused by a lack of NH_4^+ due to the lower translocation of NH_4^+ towards the shoots observed in the ^{15}N-uptake experiment (s. 4.3.2). Similar percentages of *de novo* synthesis with both N sources in the roots, implying that the effect is only present in the leaves. Interestingly, a similar difference between the treatments was also observed in C_3 leaves after 48 h in the case of Ser, Gly, Ala and Asp. This indicates that the assimilation of NH_4^+ in the C_3 species might also be limited by the availability of C skeletons or by the translocation of NH_4^+ to the shoots. This matches the low translocation rates of NH_4^+ observed in both species in the ^{15}N uptake experiment (s. 4.3.2.) Overall, *de novo* synthesis rates of most amino acids were similarly affected by the difference in N source. However, Glu and Asp were most strongly affected by the reduced synthesis rates in response to NH_4^+ in the C_4 leaves. While they are the most abundant amino acids in both species, Asp and Glu are essential for C_4 photosynthesis. Their metabolism is therefore closely connected to the photosynthetic pathway in the leaves. However, NH_4^+ assimilation seems to be predominantly located in roots in the C_4 species.

6. Conclusion and outlook

C_4 plants are less affected by the N deficiency conditions than close C_3 and C_3C_4 relatives. A lower N requirement owed to a reduced RuBisCO demand might contribute to the higher N deficiency tolerance in the C_4 *Cleome* species. This could be verified by quantifying RuBisCO content in C_3 and C_4 plants grown in normal and low N conditions (Kubien, Brown and Kane, 2011). Furthermore, assays measuring RuBisCO activity could be used to check whether the observed decrease in CO_2 assimilation rates under N deficiency conditions is correlated to a decrease in RuBisCO activity (Sales and Bernardes, 2020). Our findings suggest that this improved N deficiency tolerance of the C_4 *Cleome* species is related to a higher accumulation of NO_3^- and amino acids in leaves, which might serve as a N reserve for production of photosynthetic enzymes to upkeep photosynthesis rates under low N conditions. This hypothesis could be confirmed by establishing grafts between the C_3 and C_4 *Cleome* species and checking for recovery of amino acid or NO_3^- transport between roots and shoots under low N conditions. Furthermore, grafting C_4 shoots on to C_3 roots or vice versa, would further allow to determine which organ controls this difference in N distribution (Newell *et al.*, 2010). Furthermore, virus-induced gene silencing (VIGS) could be used to downregulate various NO_3^- and amino acid transporters in the C_4 *Cleome* species to identify candidates that affect N distribution (Carey *et al.*, 2021). The results of the metabolite profiling demonstrate a strong metabolic connection between N availability, the regulation of the TCA cycle, and amino acid metabolisms in both species. Significant differences in accumulation patterns of sugars, amino acids and TCA intermediates between the C_3 and C_4 species under N deficiency, however, suggest a differential regulation of N deficiency responses in C_3 and C_4 species. Many of the accumulating compounds can be used as respiratory substrates and N deficiency has been shown to affect the respiratory carbon pool (Lehmeier *et al.*, 2010). Thus, it would be interesting to whether dark respiration is differently affected in C_3 and C_4 *Cleome* species by measuring respiration rates using an IRGA (Fonseca *et al.*, 2021). In case of differences ^{13}C isotope labelling could be used to investigate changes in respiratory C pool in both species. Increased production of secondary metabolites via the shikimate pathway under low N conditions was found only in the C_3 species suggesting higher stress levels than in the C_4 species reinforcing the conclusion of an improved N deficiency tolerance of the C_4 species.

Measurements of ^{15}N uptake and gene expression analysis showed clear differences in the regulation of the uptake and assimilation of NO_3^- and NH_4^+ between the C_3 and C_4 *Cleome* species. However, due to the low number of replicates, the ^{15}N uptake experiment should be repeated to confirm the results. In the course of this, the inclusion of more time points could give a higher temporal resolution of the uptake process. Inhibition of photosynthesis by NH_4^+ toxicity was only observed in *C. gynandra*, while

C_3 photosynthesis benefited from NH_4^+ supplementation, confirming the hypothesized higher NH_4^+ sensitivity of the C_4 species. However, the mechanism by which NH_4^+ supplementation reduces photosynthesis rates in the C_4 species is unknown. The mechanisms responsible for alleviating NH_4^+ toxicity in the C_3 species should be further investigated e.g. through expression analysis of more AMTs and other genes known to be involved in NH_4^+ tolerance in other species (Zheng *et al.*, 2015). The higher NH_4^+ sensitivity in C_4 plants could be linked be also linked to their lower NH_4^+ uptake rates. The ^{15}N labelling experiment also showed differences in the distribution of NO_3^- and NH_4^+ between roots and shoots in the C_3 and C_4 species. Accumulation of N in roots in N-deficient plants supplied with NH_4^+ is consistent with NH_4^+ mainly being assimilated in roots in both species. The sensitivity of the C_4 species to NH_4^+ might thus be related to assimilation of NH_4^+ in roots being less efficient in C_4 plants. This hypothesis could be verified by grafting C_4 scions onto C_3 roots and comparing the effect of NH_4^+ nutrition on biomass and photosynthetic efficiency to those of non-grafted C_3 and C_4 controls. This experiment could be complemented by measuring NH_4^+ content in roots and shoots of using ion chromatography (IC) to see whether NH_4^+ is differently distributed between roots and shoots in either species or root-scion combination.

Analysis of biomass and anion content of intermediate species from the *Moricandia* and *Diplotaxis* genus did not show uniform patterns in response to both N deficiency and high NH_4^+ concentrations, which could potentially be attributed to mechanistic differences between type I and II C_3C_4 photosynthesis. A recent analysis of gene regulatory networks (GRNs) in *Flaveria* species suggest that the N metabolic pathway of type II C_3C_4 species differs from type I C_3C_4 species and might represent an alternative evolutionary solution to the ammonia imbalance in C_3C_4 intermediate species instead of a preadaptation to C_4 photosynthesis (Amy Lyu *et al.*, 2023). So far, photosynthetic efficiency was only measured in relation to N deficiency in the *Moricandia* species. Therefore, to be able to determine the full extent of the differences in the N metabolism between the different C_3C_4 intermediates in our study, C assimilation rates should also be measured in the *Diplotaxis* species and in both genera in response to high NH_4^+. Depending on whether further differences in the effect of N deficiency and NH_4^+on photosynthetic efficiency are observed, it would then be interesting to explore the mechanisms underlying the observed physiological differences using metabolic profiling. This would then allow us to compare metabolic profiles of all photosynthesis types and to potentially correlate the accumulation of certain metabolites to improved N deficiency or NH_4^+ tolerance, while also giving valuable insights into the role of N metabolism in the evolution of both C_3C_4 an C_4 photosynthesis.

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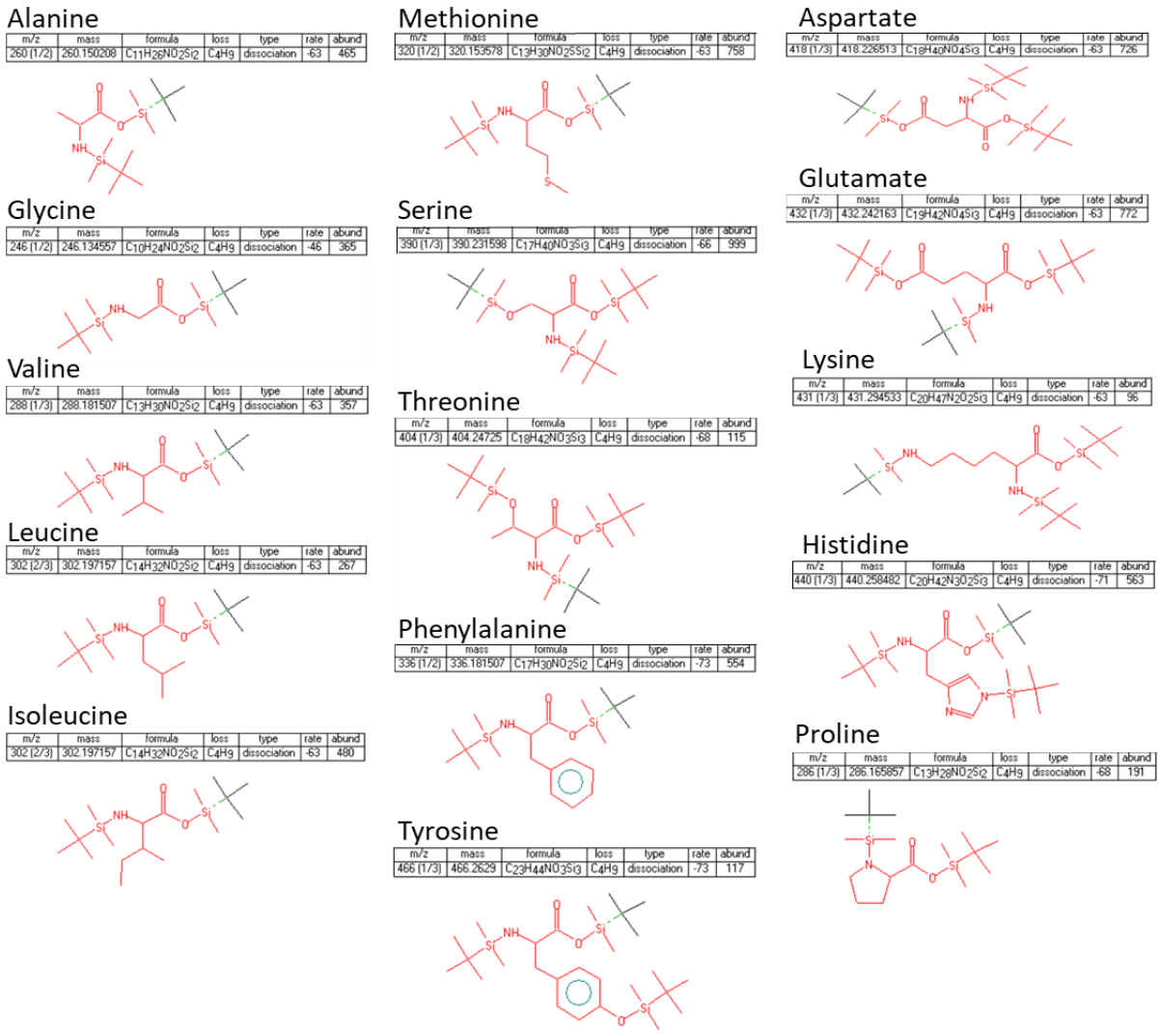
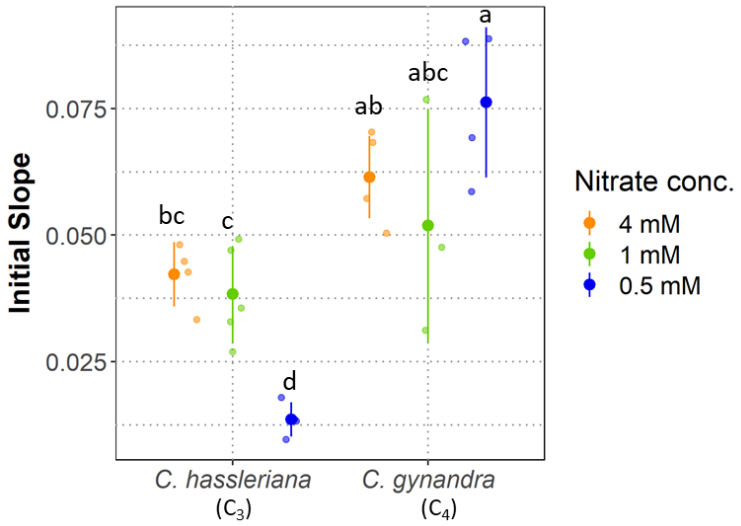
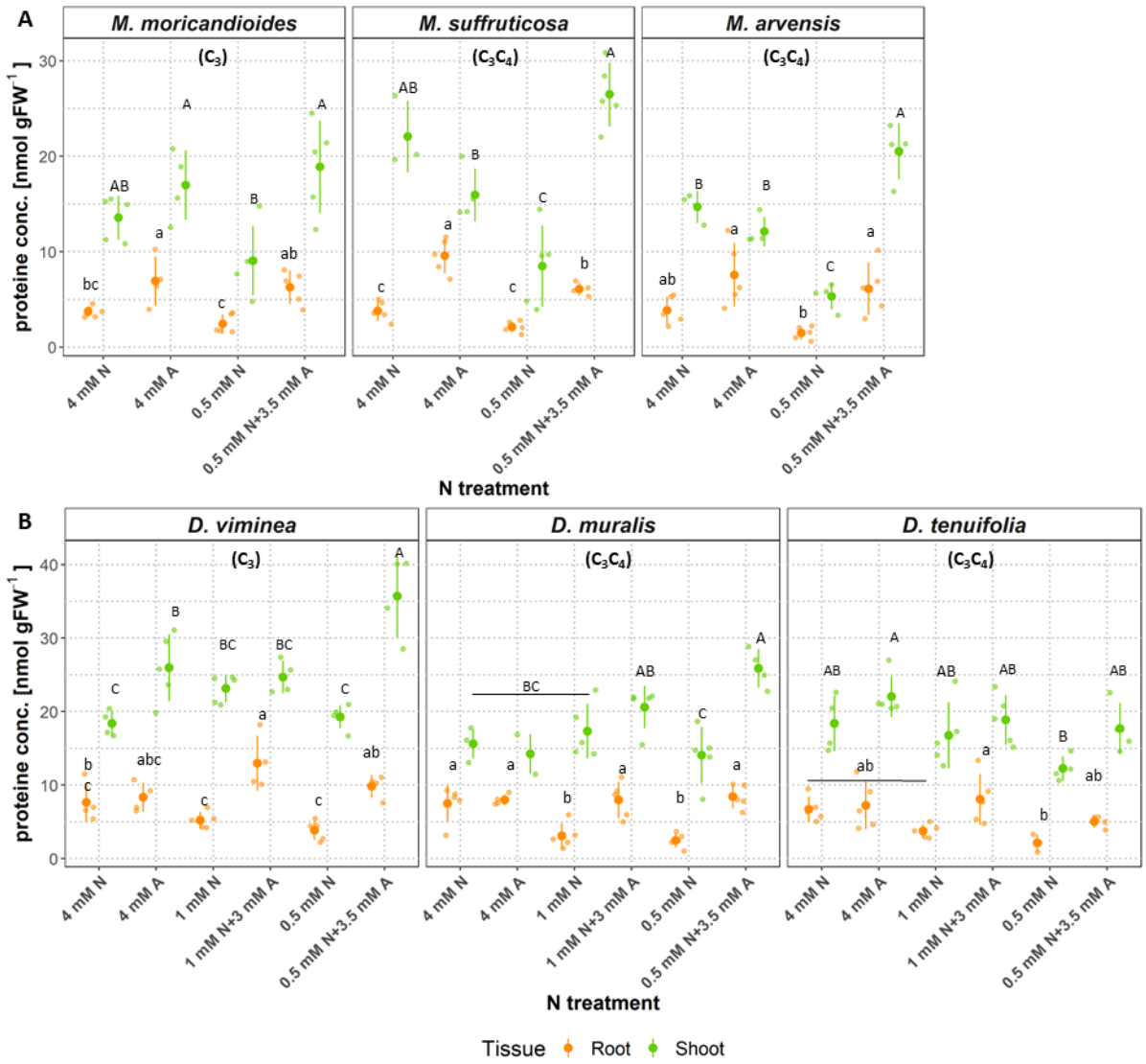
Supplement



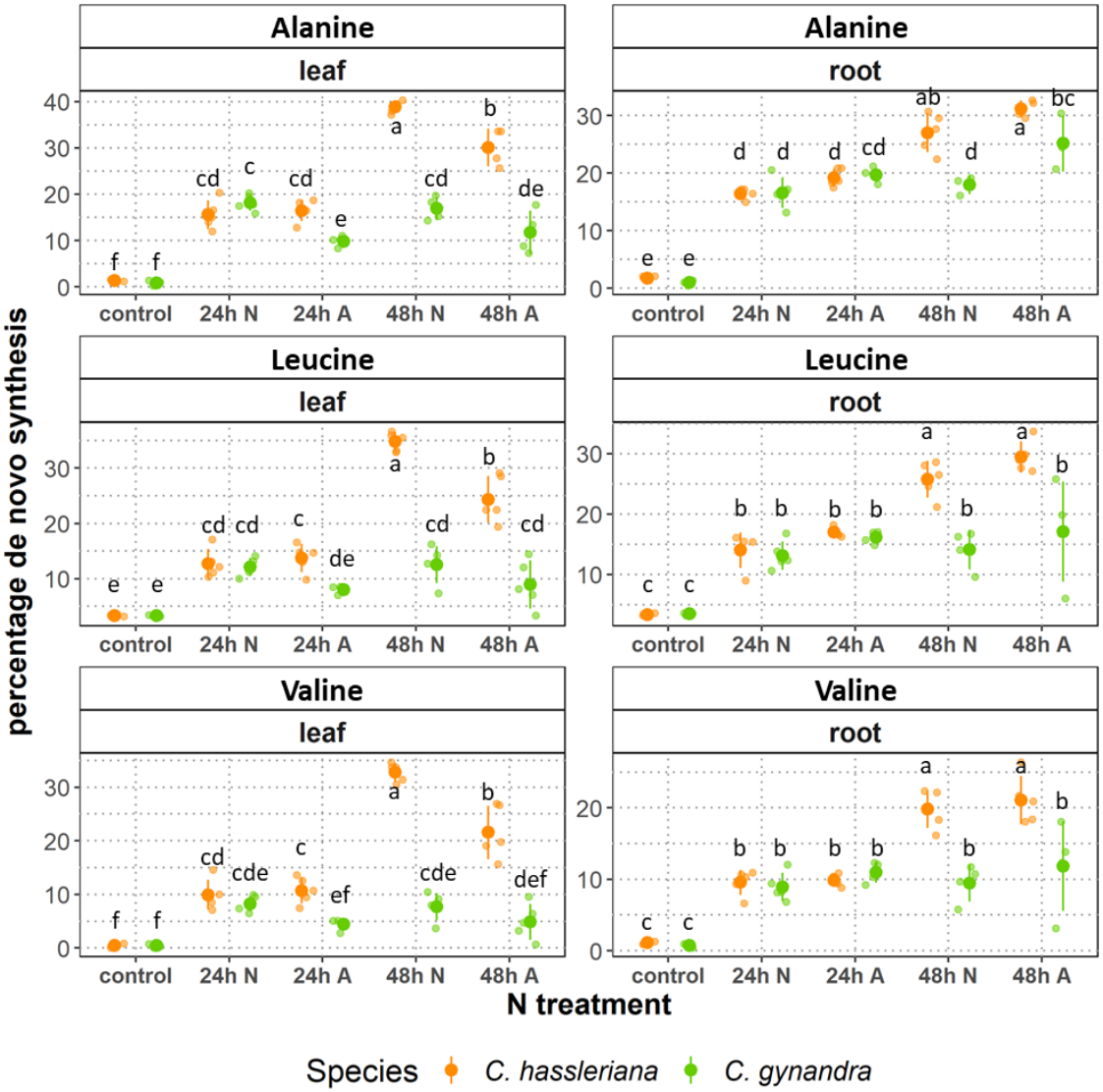
Supplemental Figure 1: TBDMS-Fragments of amino acids analysed in protein-based GC-MS Isotopologue Profiling.



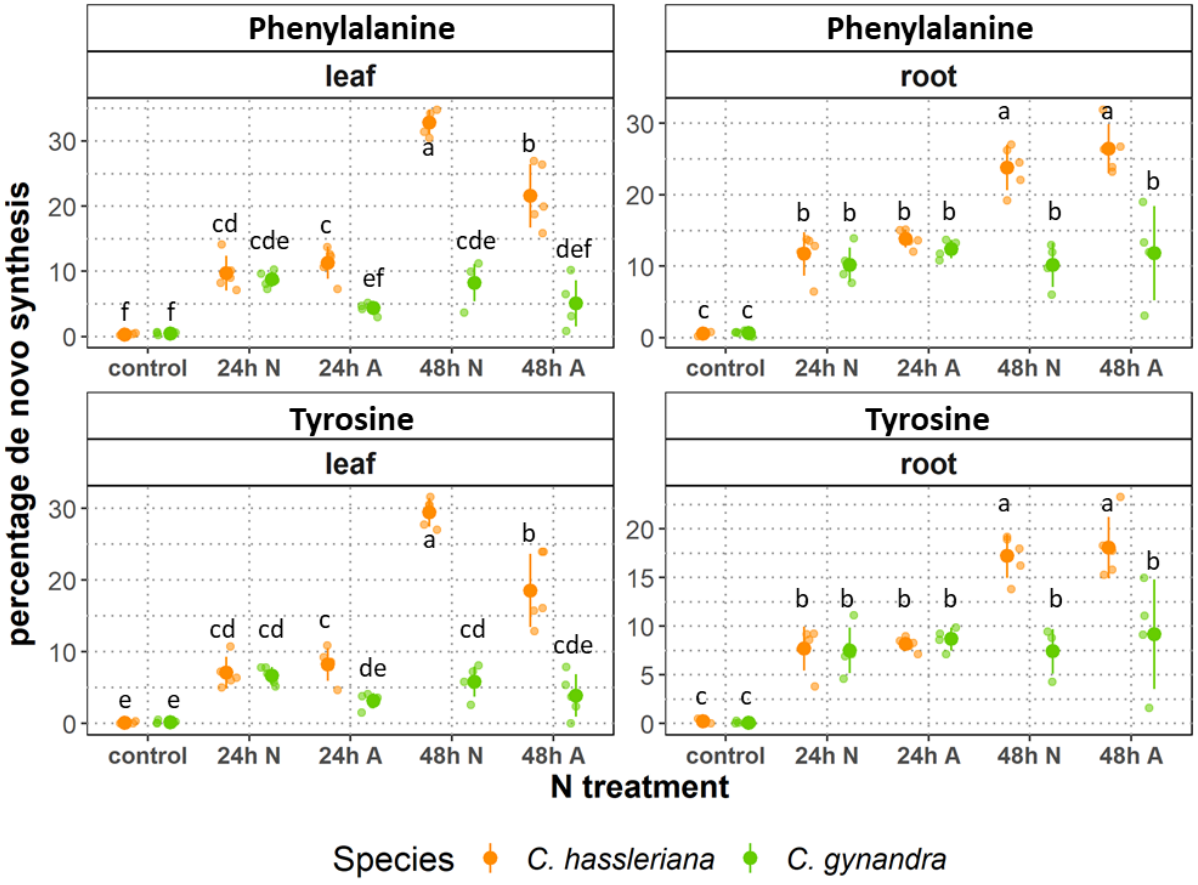
Supplemental Figure 2: Comparative gas exchange measurements revealed a significant reduction of the photosynthesis rate in C_3 but not in C_4 species in response to N deficiency. Initial slopes (mesophyll conductance) of *C. hassleriana* (C_3) and *C. gynandra* (C_4) plants grown on Hoagland solution with different nitrate concentrations (4 mM, 1 mM, 0.5 mM) (n=5).



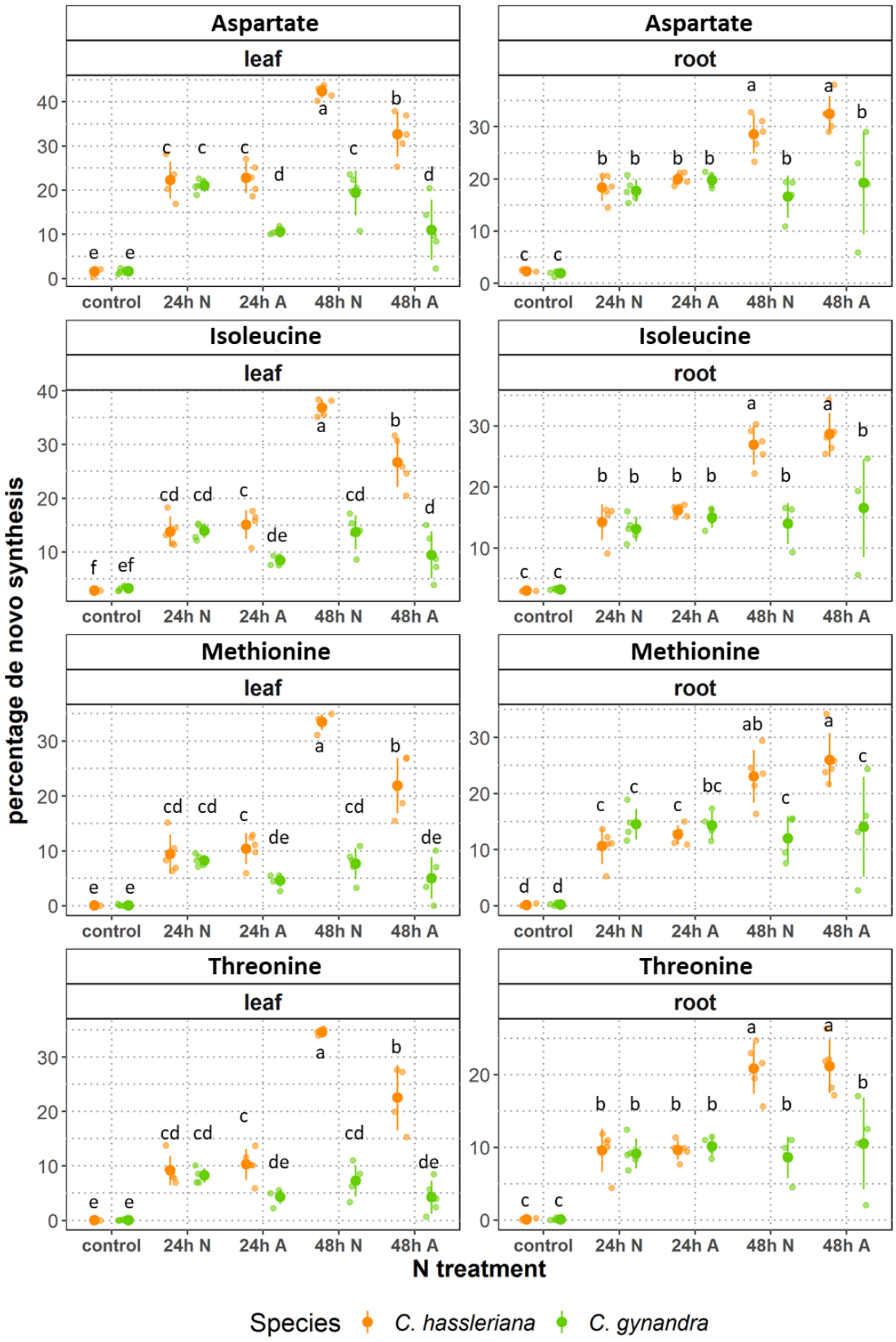
Supplemental Figure 3: Total soluble protein concentration of (A) *M. moricandioides* (C_3), *M. suffruticosa* (C_3C_4), *M. arvensis* (C_3C_4) and (B) *D. viminea* (C_3), *D. muralis* (C_3C_4), *D. tenuifolia* (C_3C_4) grown in nutrient solution with different compositions of nitrate and ammonium as nitrogen sources. Plants were bottom watered with Hoagland solution with different compositions including (1) nitrate only (4 mM N), (2) ammonium only (4 mM A), two levels of N deficiency (3) 1 mM N, (5) 0.5 mM N and two treatments substituted with ammonium to reach a combined concentration of 4 mM: (4) 1 mM+ 3 mM A, (6) 0.5 mM N+ 3.5 mM A. Protein content was measured after 2 weeks. (n=6)



Supplemental Figure 4: Percentage of *de novo* synthesized pyruvate-derived amino acids alanine, leucine and valine in leaves and roots of *C. hassleriana* (C_3) and *C. gynandra* (C_4) after re-supply with nitrate or ammonium. Plants were bottom watered with 1 mM Hoagland solution for 2 weeks before being transferred to $^{15}NO_3^-$ and $^{15}NH_4^+$ nutrient solution. respectively. Samples for measuring incorporation of ^{15}N into amino acids were taken 24 h and 48 h after transfer to either nutrient solution. The letters A and N after the timepoints indicate ammonium and nitrate nutrient solution, respectively. (n=4)



Supplemental Figure 5: Percentage of *de novo* synthesized phenylalanine and tyrosine in leaves and roots of *C. hassleriana* (C_3) and *C. gynandra* (C_4) after re-supply with nitrate or ammonium. Plants were bottom watered with Hoagland medium 1 mM for 2 weeks before being transferred to $^{15}NO_3^-$ and $^{15}NH_4^+$ medium. respectively. Samples for measuring incorporation of ^{15}N into amino acids were taken 24 h and 48 h after transfer to either medium. The letters A and N after the timepoints indicate ammonium and nitrate medium, respectively. (n=4)



Supplemental Figure 6: Percentage of *de novo* synthesized aspartate and aspartate-derived amino acids isoleucine, methionine and threonine in leaves and roots of *C. hassleriana* (C_3) and *C. gynandra* (C_4) after re-supply with nitrate or ammonium. Plants were bottom watered with Hoagland medium 1 mM for 2 weeks before being transferred to $^{15}NO_3^-$ and $^{15}NH_4^+$ medium. respectively. Samples for measuring incorporation of ^{15}N into amino acids were taken 24 h and 48 h after transfer to either medium. The letters A and N after the timepoints indicate ammonium and nitrate medium, respectively. (n=4)

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