Effect of drought and defoliation on plant growth, symbiotic nitrogen fixation, soil nitrogen availability and soil microbial dynamics in forage legumes

by

Danielito Laron Dollete

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Plant Science

Department of Agricultural, Food and Nutritional Science
University of Alberta

Abstract

Forage legumes play a crucial role in agriculture due to their symbiotic nitrogen fixation (SNF) ability by forming a symbiotic relationship with soil rhizobia, providing high-quality forage to livestock and improving soil health. The changing global climate is predicted to increase the frequency and intensity of drought, a known stress factor that negatively impacts forage legumes. Furthermore, forage legumes frequently undergo defoliation stress through pests and grazing in managed agricultural systems, which changes the source-sink relationship between above-ground and below-ground tissues. We hypothesize that the influence of drought and defoliation can potentially influence root nodulation, plant growth, SNF, and nitrogen rhizodeposition in forage legumes. In addition, the changes in plant physiology and biochemical processes induced by these stress factors can influence the soil microbiome and enzyme activities. The main objective of this project is to evaluate the effects of drought and defoliation stresses on plant growth, plant physiological responses, SNF, and their consequential influence on soil enzyme and microbial dynamics in forage legumes. Two separate greenhouse pot experiments were conducted using alfalfa (*Medicago sativa* L.) and red clover (*Trifolium pretense* L.) to evaluate the effect of drought and defoliation stresses on the above parameters. Alfalfa and red clover seedlings were inoculated with *Sinorhizobium meliloti* 1021 and *Rhizobium leguminosarum* biovar *trifolii* Mj43, respectively, and were grown until the flowering stage before applying drought and defoliation treatments. Drought study was conducted by maintaining soil moisture at 20% field capacity (FC) (severe drought), 40% FC (moderate drought), and 80% FC (well-watered) for three weeks. Defoliation treatments were simulated by trimming half of the above-ground biomass (mild defoliation) or leaving only 2 cm stubble under severe defoliation treatment. Overall, drought and defoliation significantly reduced nodulation in alfalfa and red clover. Drought negatively affected both shoot

and root biomass, while defoliation only negatively influenced root biomass while improving the final total shoot biomass. Drought was also found to reduce SNF, while SNF was reduced only in red clover following defoliation. On the other hand, soil available nitrogen was increased following severe drought stress and defoliation. In addition, drought significantly reduced N-acetyl-glucosaminidase and β-D cellobiosidase enzyme activities in alfalfa and red clover soil, respectively. On the contrary, defoliation positively influenced β-1, 4-glucosidase, β-D-cellobiosidase, and phosphatase enzyme activities in soil under both forage legumes. Lastly, microbiome data showed shifts in the relative abundance of some key bacterial taxa under drought and defoliation stresses. Overall results suggest that drought and defoliation induced varied influences on SNF and plant growth, eliciting different effects on nutrient cycling enzyme activities, soil nitrogen availability, and shifts in soil microbial diversity.

Keywords: forage legumes, drought, defoliation, nodulation, symbiotic nitrogen fixation, soil enzymes, available soil nitrogen, soil microbiome

Preface

Author Contributions are as follows:

Chapter 2 - Danielito Dollete, Malinda Sameera Thilakarathna, and Cameron N Carlyle conceptualized and designed the research; Danielito Dollete performed the experiments and collected data. Malinda Sameera Thilakarathna, Cameron N Carlyle, and Krzysztof Szczyglowski provided resources; Danielito Dollete, Rhea Amor Lumactud, and Brett Hill performed formal data analysis; Danielito Dollete and Rhea Amor Lumactud did validation and visualization; Danielito Dollete wrote the original draft. All authors contributed to the review and editing of the manuscript. Malinda Sameera Thilakarathna acquired the funding.

Chapeter 3 - Danielito Dollete, Malinda Sameera Thilakarathna, and Cameron N Carlyle conceptualized and designed the research; Danielito Dollete performed the experiments and collected data. Malinda Sameera Thilakarathna, Cameron N Carlyle, and Krzysztof Szczyglowski provided resources; Danielito Dollete, Rhea Amor Lumactud performed formal data analysis; Danielito Dollete and Rhea Amor Lumactud did validation and visualization; Danielito Dollete wrote the original draft. All authors contributed to the review and editing of the manuscript. Malinda Sameera Thilakarathna acquired the funding.

Chapter 2 of this thesis has been published as, Dollete, D., Lumactud, R. A., Carlyle, C. N., Szczyglowski, K., Hill, B., Thilakarathna, M. S. (2023) Effect of drought stress on symbiotic nitrogen fixation, soil nitrogen availability and soil microbial diversity in forage legumes. Plant and Soil 491: 1573-5036

Acknowledgment

I want to extend my heartfelt gratitude to supervisors Dr. Malinda Thilakarathna, my collaborator and committee member Dr. Cameron Carlyle, and Dr. Rhea Lumactud my mentor and "Ate". This experience has been a time of expansive personal growth, and I am deeply grateful for their unwavering encouragement, patience, and willingness to share their expertise. Any success I've achieved and will achieve will be fruits in large part of your guidance. Special thanks to Krzysztof Szczyglowski and Brett Hill for their valuable contribution for to the two research chapters.

To my first and most trusted friend, Dilrukshi Liyanage, here in Canada. Firstly, thank you for your support in my first project. Most of all, thank you for our time together, how brief it may be, you made me feel I rightfully deserve to be here and understand my ups and downs. You do not know how much it meant to me.

I would also like to take this opportunity to thank my friends in Italy and especially in the Philippines who have truly loved me unconditionally and unwaveringly encouraged me from college until now. Thank you for being there for me when my thoughts was out to bully me, when doubt crept in my mind. I appreciate our sleepless nights just talking about anything, bonding over our downs and mental health hahaha! Honestly could not ask for a better circle of friends.

Lastly, and definitely not the least. Thank you to the Department of Science and Technology Science education Institute of the Philippines and University of Alberta. Without the scholarship they have provided, aspiring international researchers such as I was before would not get the opportunity to experience the beauty and excellence that Alberta and the university has to offer. The comfort of not thinking about where to get our living expenses tremendously helped me and my co-scholars be focused on finishing our respective degrees.

Table of contents

List of figures

List of abbreviations

ΦII	Quantum yield
ΦNPQ	Non-photochemical quenching
ABA	Abscisic acid
ANOVA	Analysis of variance
BNF	Biological nitrogen fixation
C	Carbon
Cello	β-D cellobiosidase
$C_2H_3NaO_2$	Sodium Acetate
CID	Carbon isotope discrimination
DWT	Dry weight
EEA	Extracellular enzyme activity
FC	Field capacity
FDR	False discovery rate
Glu	β-1, 4-glucosidase
IRMS	Isotope Ratio Mass Spectrometer
KOH	Potassium hydroxide
LEF	Linear electron flow
MAS	Marker-assisted selection
MDH	Malate dehydrogenase
MUB	Methylumbelliferone
N	Nitrogen
N_2	Dinitrogen
%Ndfa	Percentage of nitrogen derived from atmosphere
NAG	N-acetyl-β glucosaminidase
NaOCl	Sodium hypochlorite
NH_4^+	Ammonium

NO_3^-	Nitrate
O_2	Oxygen
OTU	Operational taxonomic unit
P	phosphate
PC	Principal component
PCA	Principal component analysis
PERMANOVA	permutational analysis of variance
Phos	Phosphatase
PRS	Plant root simulator
RDP	Ribosomal database project
RWC	Relative water content
SNF	Symbiotic nitrogen fixation
SOC	Soil organic compounds
SPAD	Soil plant analysis development
WUE	Water-use efficiency

Chapter 1 – Literature review

1.1. Importance of legumes in agriculture

Effective management of nitrogen (N) in the cropping system has become a priority worldwide due to the risk of environmental pollution linked with synthetic N fertilizer. The excessive use of inorganic N fertilizer can lead to nitrate leaching into the groundwater (Camargo and Alonso, 2006) and an increase in greenhouse gas emissions due to denitrification (Follett and Delgado, 2002). Consumption of contaminated water will inevitably cause serious health problems for both humans and animals. These issues bring about the popularity of conservative sustainable agriculture practices. In sustainable agriculture, there is greater interest in using legumes as an alternative N source through biological nitrogen fixation (BNF) for achieving sustainable crop and livestock production and ecosystem services (Rochon et al., 2004). Legumes contribute by fulfilling multiple services:

(i) Food-system level: Both humans and livestock rely on legumes as a cheap source of plant-based proteins (Tharanathan and Mahadevamma, 2003). Legumes are among the world's most cultivated crops globally, third only to cereals and oilseeds (Popelka et al., 2004; Gogoi et al., 2018). They can be divided into two groups based on their capacity to be grown in different seasons, mainly cool-season and warm-season legumes (Sita et al., 2017). Species of this family are of paramount importance in nutritional security, especially in developing countries, where legumes are considered the "poor man's meat" as a significant source of protein and other nutrients (Tharanathan and Mahadevamma, 2003; Gogoi et al., 2018; Nadon and Jackson, 2020). The average protein content of legumes varies between ~18-25% depending on the genotype, growth environment, and cultural practices (Tharanathan and Mahadevamma, 2003; Singh, 2010). Legumes are also rich in biopeptides, copper, cysteine, folic acid, isoflavonoids, lecithin, manganese, methionine, phosphorus, potassium, threonine, tocopherols, tryptophan, zinc, and other fatty acids (Singh, 2010; Meena and Lal, 2018).

(ii) Production level: Nitrogen-rich plants such as forage legumes and pulses are usually used in crop rotations to supply N (Chalk, 1998). This context allows legumes suited for inclusion in low-input and low-greenhouse gas emission systems (Lemke et al., 2007; Farooq et al., 2016; Gogoi et al., 2018). Legume's ability to fix N_2 biologically in symbiosis with soil rhizobia contributes to its importance for crop rotation to fulfill the N requirement for succeeding crops, thus improving soil

quality and sustaining environmental balance (Giller, 2001; Courty et al., 2014; Sita et al., 2017; Gogoi et al., 2018). In addition, legumes produce high amounts of proteins because of their capacity to fix atmospheric N_2, which is then assimilated into amino acids and proteins (Nadon and Jackson, 2020). Nitrogen fixed by forage legumes under legume/grass systems ranges from 13 to 682 kg N ha^{-1} yr^{-1} (Ledgard and Steele, 1992; Wagner, 2011).

(iii) Cropping system level: Legumes are used in crop rotations to play a role as diversification crops based on systems dominated by a few notable species such as clovers, alfalfa, vetches, and grain legumes such as soybeans and peas. A diverse line of crops breaks pest and disease cycles, improving soil microbial community structures for crop defence and contributing to nutrient cycling and nutrient supply, reducing inputs in nutrient-deficit soils (Emerich et al., 2009; Köpke and Nemecek, 2010; Voisin et al., 2013).

1.2. Biological Nitrogen Fixation

1.2.1. Biological nitrogen fixation in forage legumes

Nitrogen is an essential macronutrient for growth and development in living organisms. It is a key element in the nucleic acids DNA and RNA, which are biological molecules that dictate the form and functions of all living things. In plants, it plays an important role in growth: a dearth of N leads to stunted growth, leading to low yield; but an excessive amount of N can be toxic to plants (Britto and Kronzuker, 2002). Thus, N is one of the most limiting factors for plants. Nitrogen is, in fact, most abundant in the Earth's atmosphere. However, although 78% of the earth's atmosphere contains N_2 gas, it is not in available form due to the strong triple bonds between the two N atoms (Graham and Vance, 2000; Kumar et al., 2020). Furthermore, it is one of the most critical limiting nutrients in the soil for crop production (Soumare et al., 2020). Some prokaryotic organisms have the ability to fix atmospheric N_2 in tandem with an anaerobic enzyme called nitrogenase. Nitrogenase is a multimeric protein complex that is composed of two proteins of differing sizes: molybdoferredoxin (Mo-Fe) protein (dinitrogenase) and azoferredoxin (Fe) protein (dinitrogenase reductase) (Kumar et al., 2020). Biological N fixation is an energy-intensive process requiring 16 high-energy adenosine triphosphate (ATP) to reduce each mole of N_2 into two NH_3. An additional 12 ATP molecules are used to assimilate and transport plant-usable nitrogen form, totaling 28 ATP molecules. The stoichiometry of biological N fixation under optimum conditions can be expressed as:

$$N_2 + 8H^+ + 8e^- + 16\ ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16\ P_i$$ (Kumar et al., 2020)

Some N_2-fixing bacteria are free-living, forming the non-symbiotic category of N-fixation systems, some are associative fixers, and others form symbiotic relationships. One of the prominent symbiotic forms of N fixation is legume-rhizobia symbiosis. Legume plants form knob-like structures in roots called nodules that house the N-fixing bacteria. Root nodules are formed from the successful infection of the root cortex by N-fixing bacteria such as *Rhizobium*. The enzyme nitrogenase previously described is highly sensitive to oxygen (O_2), whereas the symbiotic bacteria are strictly aerobic. Legumes have developed two adaptations to protect nitrogenase from O_2 while supplying adequate O_2 for rhizobia in root nodules: the formation of the oxygen diffusion barrier into the nodule and the production of oxygen carrier proteins called leghaemoglobin (Gordon et al., 2001; Hossain et al., 2017).

1.2.2. Nodulation process

Plant root nodule houses the bacteroid responsible for specific plants like legumes to be able to fix atmospheric N_2. This root organ is formed through the symbiosis of legumes with the rhizobia bacteria. The establishment of this mutualistic relationship begins with the signal exchange mechanism orchestrated by the rhizobia and host plants through flavonoids and isoflavonoids secreted by the host plant in the rhizosphere, recognized by compatible rhizobia species. Germinating seeds or roots secrete specific flavonoids that interact with the rhizobia NodD bacterial protein family (Maj et al., 2010; Cesco et al., 2010). Multimers of NodD will bind to the promoter regions and induce the expression of nodulation genes (*nod* genes) (Jones et al., 2007). Once the nod genes are activated, the bacteria produce specific lipochito-oligosaccharides (*nod* factors) to induce rhizobia-to-plant signal exchange (Kondorosi et al., 2013).

Nod factors are essential signalling molecules produced by the products of these nod genes. Nod factors usually consist of three to five 1,4 N-acetyl-D-glucosamine with a long lipid acyl group attached to the nonreducing end of the backbone (Gage, 2004). Nod factors from different rhizobia species have been identified. They differ in terms of the length and saturation of the acyl group, the number of glucosamine residues, and the nature of specific modifications on the backbones (Dénarié et al., 1996; Downie, 2005). These variations allowed the species specificity that is observed in this symbiosis.

Nod factors are perceived by the host plant by specific LysM receptor kinases in the root epidermal cells at concentrations as low as 10^{-12} M (Oldroyd and Downie, 2004). The binding of these nod factors to the LysM domains results in the downstream genetic and metabolic signalling cascades mediated, in part, by the rapid influx of Ca^{2+} (Charpentier and Oldroyd, 2013). This cascade induces many plant responses, such as root hair deformation and pre-infection structures, to trap the rhizobia in the cell wall (Gage, 2004). This close association allows the directional supply of signalling molecules from the bacteria to signal the formation of an infection thread that grows and directs the rhizobia toward the inner root cortex (Jones et al., 2007). The signalling cascades also result in the increased division of the cortical cells, leading to composite structures forming the nodule primordium (Gage, 2004). The infection thread allows the bacteria to penetrate the dividing cortical cells (Oldroyd, 2013). Inside the infected cells, the rhizobia begin replication. While replicating, they are encapsulated by the plant-derived membrane called the peribacteriod membrane (PBM), forming a facultative organelle called symbiosome. The bacteria then differentiate into nitrogen-fixing endosymbiotic bacteroids (Jones et al., 2007; Oldroyd, 2013).

1.2.3. Nitrogen fixation and nitrogen exportation

Symbiotic interactions between the leguminous plants and compatible rhizobia play a crucial role in the soil-N pool of agroecosystems, promoting sustainability. This nodule symbiosis allows legumes access to atmospheric N_2 and converts them into bio-assimilable forms of N, ammonia (NH_3), or ammonium (NH_4^+) (White et al., 2007; Udvardi and Poole, 2013). Aside from allowing legumes to fix N_2, legumes are able to produce and store high amounts of proteins since amino acids require biological N sources (Nadon and Jackson, 2020). This context underlines the importance of symbiotic nitrogen fixation (SNF) in enabling legumes to provide protein-rich food sources for humans in developing countries and animal feed in well-to-do sectors of society (Graham and Vance, 2003). Through SNF, legumes are enabled to deposit organic fertilizer into the soil through above-ground litter decomposition and rhizodeposition via root exudates (Thilakarathna et al., 2016).

Inside the nodule, the resulting ammonia/ammonium is assimilated mainly into the amides (e.g. asparagine and glutamine, or as the ureides (e.g., allantoin and allantoic acid)) (Atkins et al., 1982; White et al., 2007). The saturation of assimilated amino acids within the nodule results in negative feedback affecting N fixation. In order to maintain N fixation, amino acids need to be

exported to other deprived plant tissues (Carter and Tegeder, 2016). After synthesis, amino acids are transported from the nodule via the xylem into the shoot (Atkins et al., 1982; Tegeder, 2014). Amides are the dominant N form transported in legumes categorized as amide exporters. In contrast, ureides are the main N form exported by ureide-exporters (Unkovich et al., 2008). Typically, legumes originating in the temperate regions (e.g., alfalfa, peas, lentils) are amide-exporters, whereas those originating in the tropics and subtropics (e.g., soybeans, cowpeas) dominantly export the ureide N transport forms (Atkins and Smith, 2007; Unkovich et al., 2008; Tegeder, 2014).

1.3. Nitrogen rhizodeposition

1.3.1. Rhizosphere

The soil is described as a stimulating living environment that provides life-sustaining nourishment and ecosystem services to support the diversification of macro- and micro-fauna and flora (Doran et al., 2000; Idowu et al., 2020). The rhizosphere, first described in 1804 by Lorentz Hiltner, originates in part from the Greek word "rhiza," which means root (McNear Jr., 2013). The rhizosphere is the environment immediately near the vicinity of the plant's roots. This environment is rich in nutrients readily available in the soil and nutrients released by the plant through its roots. This environment is influenced by the chemicals excreted by plant roots and the activities of microbial communities in the particular microzone (Koo et al., 2005). The rhizosphere has been further divided into three different zones. The endorhizosphere is the innermost portion of the rhizosphere that includes a portion of the root cortex. The endodermis is where microorganisms occupy the "free spaces" between cells called the apoplastic space. The rhizoplane is the median section of the rhizosphere, where it involves the root surface and the adjacent soil and exudates. The ectorhizosphere is the portion that extends from the rhizoplane into the soil surrounding the roots. Rhizosphere cannot be directly qualified by size or shape but instead quantified via analyzing gradients of chemical, biological, and physical attributes that change proportionately as the roots grow.

1.3.2. Nitrogen rhizodeposition pathways

Rhizodeposition is the release of compounds usually distinguished as root exudates into the surrounding soil (Walker et al. 2003), first coined to describe carbon (C) release (Lynch and

Whipps, 1990). Nitrogen rhizodeposition explicitly details N movement into the soil, which underlies the role of rhizodeposition in below-ground N transfer from legumes to non-legumes in the agroecosystems. Considering less N than carbon is released through rhizodeposits, the composition and amount of released compounds vary between legume species and even in their life stages (Bais et al., 2006), which are also affected by the growth conditions (McNeill et al., 1997). The potential N rhizodeposition pathways are root exudation and N release through the decomposition and senescence of below-ground tissues (Fig. 1.1) (Wichern et al., 2008; Fustec et al., 2010).

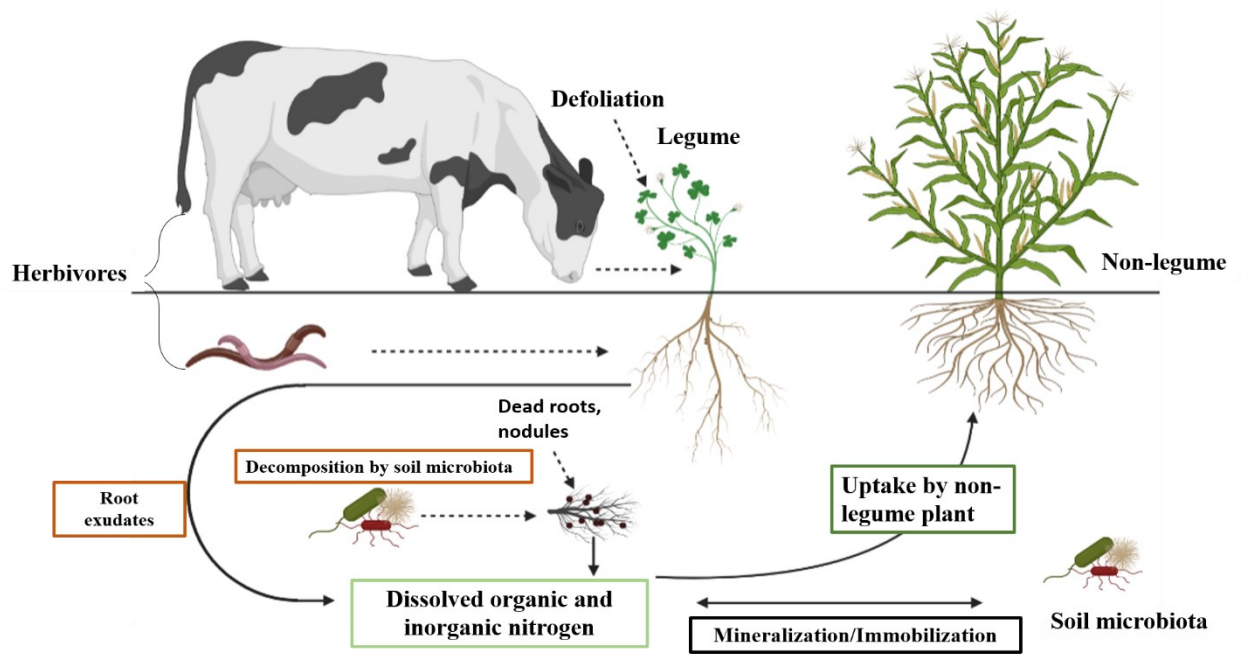


Figure 1.1 Several below-ground nitrogen rhizodeposition mechanisms in legumes (root exudates and decomposition of roots and nodules).

1.3.2.1. Nitrogen rhizodeposition through decomposition and senescence of root and nodule tissues

Senescing root and nodule tissues are a significant source of fixed N transferred into the soil (Fustec, 2010). No reliable quantitative methodologies distinguish N transfer due to decay and senescence from exudated N forms. The decomposition of plant tissues is the top and main pathway of N rhizodeposition (Dubach and Ruselle, 1994; Sierra et al., 2007). Nevertheless, N

rhizodeposition may vary significantly between legume species and the tissue origin (Ta and Faris, 1987). Although little quantitative data is available, quantification of below-ground N transfer via decay of roots and nodules in legumes by Ledgard and Steele (1992) estimated that about 3 to 102 kg N ha^{-1} yr^{-1} could be transferred through this pathway. Dubach and Russelle (1994) found out that the decomposing roots of alfalfa (*Medicago sativa* L.) release more N than their nodules. An opposite trend was observed in birdsfoot trefoil (*Lotus corniculatus* L.) (Vance et al., 1979). Although tissue decay and senescence are considered the main N transfer pathways and contribute significantly to the soil N pool compared to root exudates, these pathways requires additional external help (e.g., macro and micro herbivores, microbes, enzymes) to decompose and cycle nutrients. As a result of this extra requirement, this pathway is a slow process and primarily contributes to the later plant growth stages (Louarn et al., 2015).

1.3.2.2. Nitrogen rhizodeposition through root exudates

Root exudates commonly come in the form of soluble metabolites (e.g. sugars, amino acids, and organic acids) (Canarini et al., 2019). They can be released as low-molecular-weight compounds that are easily decomposed (e.g., amino acids, organic acids, phenolics, and other secondary metabolites) (Bertin et al., 2003; Bais et al., 2006; Marschner, 2012) or high-molecular-weight compounds (e.g., enzymes, mucilage, protein) (Walker et al., 2003; Badri and Vivanco, 2009; Canarini et al., 2019). These compounds can be released actively or passively (Dennis et al., 2010) and may also include compounds released from senescing roots (Neumann and Römheld, 2007). Plants have exhibited a higher control level over high-molecular-weight compounds than low-molecular-weight compounds, but are generally considered a passive process (Phillips et al., 2004; Badri and Vivanco, 2009; Chaparro et al., 2013). Specific transporters are also involved in releasing root exudates, mainly an ATP-binding cassette and the multidrug and toxic compound extrusion families (Badri and Vivanco, 2009; Weston et al., 2012). The primary forms of low-molecular-weight N-containing compounds are ammonium and amino acids (Paynel et al., 2008; Lesuffleur et al., 2013). Root exudates contain different amino acids, including alanine, arginine, aspartate, asparagine, glutamate, glutamine, glycine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, tyrosine, and valine, with glycine and serine being the dominant forms recovered from various species like in alfalfa and clovers (Lesuffleur et al. 2007; Paynel et al., 2001, 2008). Similar to other forms of rhizodeposition, root exudation is influenced by abiotic

factors such as defoliation (Ayres et al., 2007) and biotic factors (e.g., plant species, herbivores, microbes) (Bais et al., 2006).

1.4. Soil microbiome and enzyme activity

The soil is so astoundingly abundant in microorganisms (e.g., bacteria, fungi, archaea, protozoa, viruses) that bacteria alone comprise 15% of the total living biomass on earth (Bar-On 2018). A specific microbial community termed "microbiome" was first coined by Lederberg and McCray (2001), which is defined as a "set of resident microorganisms that inhabit a given host/environment." The soil microbiome is responsible for the biogeochemical cycling of nutrients and other valuable elements necessary for biological life (Wang et al., 2020; Trasar-Cepeda et al., 2008; Kabiri et al., 2016). Much like the source-sink context in plants, soil is a major source of beneficial microbes (Dias, 1996) and, thus, a foundation of ecosystem health. In plants, particular microbial members can assemble in the rhizosphere and be preferentially recruited by the plant into its roots (Edwards et al., 2015; Finkel et al., 2017). These recruited microorganisms carry out multiple plant growth-promoting activities, including fixation (e.g., N-fixation), mineralization, solubilization (e.g., phosphate), mobilization of nutrients, stimulating the production of growth hormones (e.g., auxin and gibberellin), and generating/triggering plant disease defences (Santos et al., 2021; Trivedi et al., 2021). Most of these agendas are augmented and made possible through the release of enzymes by specific microbes (Kanté et al., 2021), which are also part of the foundation of soil health (Alkorta et al., 2003; Adentuji et al., 2020). Enzyme activities depend highly on the amount of enzyme-substrate released and resource availability (Kwiatkowski et al., 2020; Mndzebele et al., 2020; Harasim et al., 2020). It is noted that enzyme activities in the rhizosphere are higher compared with bulk soil due to the high occurrence of plant-microbe interactions that can be observed within that microecosystem (Vandana et al., 2021), making enzymes an excellent biological indicator in agriculture (Dick et al., 1997; Gianfreda et al., 2015; Egamberdieva et al., 2010).

1.5. Abiotic and biotic stress on forage legume

Based on climate predictions, the rising global temperature may and will impact the earth's hydrologic cycle, influencing drought occurrences (Sheffield and Wood, 2008). Drought is an increasing threat that will significantly negatively affect crop production (Lesk et al., 2016; Kunert

et al., 2016). On the other hand, the mismanagement of grazing in controlled pastures and the influence of uncontrollable variables involved in foliar damage result in the loss of productivity. The removal or damage of above-ground tissues causes an imbalance in resource allocation between above- and below-ground biomass (Gordon et al., 1990; Richards, 1993). Independently, defoliation and drought cause many deleterious effects not only to legume plants but also to other players related to plant function and growth.

1.5.1. Defoliation and drought on nodulation and symbiotic nitrogen fixation

Drought directly influences the initiation of the nodulation process by disturbing the communication between the soil rhizobia bacteria and the legume roots. The lack of soil moisture impedes the sending and recognition of signalling molecules, resulting in poor nodulation and reduced N fixation (Miransari et al. 2013). Secondly, drought significantly reduces the survivability and the abundance of indigenous and externally inoculated rhizobia in the soil, resulting in poor nodulation (Thilakarathna and Raizada, 2017). The nitrogenase enzyme is the key player involved in the N fixation process. However, this enzyme is susceptible to oxygen and will inevitably stop functioning when exposed to high oxygen concentrations (Sulieman and Tran, 2016). The root nodules of the legumes are capable of maintaining a microaerobic environment (Serraj et al., 1999; Serraj 2003). However, defoliation and drought stress cause the loss of turgor and cell volume, limiting oxygen diffusion and disrupting the microaerobic environment, resulting in the inhibition of nitrogenase activity (Serraj et al. 1999; Serraj 2003; González et al. 2015; Sulieman and Tran, 2016). Defoliation also directly influences the existing nodules by restricting the supply of photosynthates, inducing nodule senescence (King and Purcell, 2001). There are two known main mechanisms that result in low N fixation in legumes; oxygen limitation in nodules, and carbon shortage for respiration.

As noted previously, BNF in legume-rhizobia symbiosis requires a microaerobic environment in the nodule due to the nitrogenase enzyme's anaerobic characteristics and the nodulating bacteria's aerobic characteristic. Nitrogenase is irreversibly inactivated if exposed to high oxygen concentrations (Sulieman and Tran, 2016). The inhibition of nitrogenase activity under water deficit and defoliation is linked to oxygen limitation of nitrogenase-linked respiration (Del Castillo et al., 1994; Hartwig and Nösberger, 1994). It is suggested that during drought stress or following defoliation, the legume nodule tissues begin to senesce and cell permeability

increases, which limits the oxygen diffusion to the central zone of the nodule (Pankhurst and Sprent, 1975; Serraj et al., 1999; Serraj, 2003). Furthermore, drought conditions induce ABA synthesis (Ren et al., 2007), while defoliation increases its accumulation (Gómez-Cadenas et al., 2000; Iglesias et al., 2003), the upregulation of this hormone consequently reduces the synthesis of leghaemoglobin and dramatically increasing the oxygen diffusion resistance into the nodules (González et al. 2001).

Furthermore, the reduction of N fixation under drought and following defoliation is caused by the reduction in nodule carbon flux (Arrese-igol et al., 1999). The inhibition of nodule sucrose synthase relates to the shortage of carbon substrate essential for SNF (González et al. 2015; Kunert et al. 2016). Sucrose is the main carbon source supplied from photosynthates to the bacteroid in legume nodules to facilitate SNF. The sucrose synthase enzyme catalyzes the process of breaking down sucrose into hexose, catabolizes it next into phosphoenolpyruvate, and then finally converts it into oxaloacetate by PEP carboxylase (Sulieman and Tran, 2016). The enzyme malate dehydrogenase reduces oxaloacetate into malate, producing NAD^+ (Vance and Gantt, 1992). During drought and at defoliation, both sucrose supply and sucrose synthase activity significantly decline, affecting bacteroid respiration (González et al. 2015; Kunert et al. 2016).

1.5.2. Abiotic and biotic stress on legume growth

1.5.2.1. Drought on forage legume growth

Among abiotic factors, drought is one of the most deleterious stress factors that can affect plant growth. The loss of turgor and cell volume under drought stress is equated to cell dehydration. This reduction in plasticity under water deficit results in a reduction in cell expansion and cellular division and a reduction in water and nutrient uptake, all of which will affect leaf size, limiting stem and root growth (Kaushal and Wani, 2016). In addition, drought induces stomatal closure to conserve internal moisture, consequently reducing photosynthesis (Liu et al., 2005; Mak et al., 2014). In response, plants allocate resources for root growth to access deep-seated water (Kaspar et al., 1984), thus causing an imbalance in biomass partitioning in favor of the roots, increasing root:shoot ratio (Manavalan et al., 2009).

1.5.2.2. Defoliation on forage legume growth

The effect of defoliation of plant growth is based on the source-sink interaction between the above- and below-ground organs and tissues. Following defoliation, there is a necessity to re-allocate carbon and N sources to the remaining above-ground biomass to sustain the regrowth of photosynthetic tissues, all the while maintaining copious partitioning to root and nodule to maintain respiration and nutrient uptake (Aranjuelo et al., 2015; Xu et al., 2013). Furthermore, removing the above-ground mass constitutes the loss of photosynthetic tissues, resulting in the reduction of the plant's photosynthetic capacity. This reduction restricts the flow of photosynthates into the roots and nodules (King and Purcell, 2001), thus promoting below-ground tissue senescence, which consequently affects N availability through reduced N fixation and increased N release into the soil (Hamilton et al., 2008; Carrillo et al., 2011; Thilakarathna and Raizada, 2019).

1.5.3. Abiotic and biotic stress on soil microbiome and enzyme activity

In the context of soil-based plant production, plants are generally in close proximity to soil microbes living closely with their root system in the rhizosphere area (Berg et al., 2016). Next to the soil, the root system is the main source of nutrients for microbes associated with plants, made available through rhizodeposition and cycling by specific enzymes. One of the main roles of soil microbes is to cycle nutrients and organic matter turnover (Gattinger et al., 2008; Condron et al., 2010). They facilitate this ecological function by releasing specific extracellular enzymes to catalyze various reactions (Bowles et al., 2014; Rao et al., 2014; Adetunji et al., 2020). The main factor influencing extracellular enzyme activity is the condition and abundance of soil microbes. The strong affiliation microbes have with the root system suggests that any changes in exudation, growth patterns, and overall health of roots and nodules in response to defoliation and drought stress will unequivocally alter bacterial community structure (Preece and Peñuelas, 2016; Jones et al., 2009; Hamilton et al., 2008; Carrillo et al., 2011). Furthermore, any changes in plant biomass and physiology brought about by either defoliation or drought stress influence plant-microbe interaction (Gattinger et al., 2008; Gaiero et al., 2013; Berg et al., 2014). The manner of rhizodeposition is uniquely different from one plant species to another (Cavaglieri et al., 2009), which is further differentiated depending on the plant's growth stage and health (Chaparro et al., 2013, 2014). Exudates are the main determinant of plant-microbe interaction as they contain

limiting growth nutrients necessary for the proliferation of microbes (Kanté et al., 2021; Macdonald et al., 2006; Ma et al., 2018). However, it is also important to note that the diversity of microbes in terms of physiology and morphology can determine their survivability and their response under plant stress (Huber et al., 2022; Ma et al., 2018).

1.5.3.1. Drought on soil microbiome and enzyme activity

Drought has a profound influence on the N dynamics in both the plant and the soil. Under drought, the structural integrity of the root cell wall is weakened, increasing cell permeability and influencing the type and amount of exudates released (Brophy and Heichel, 1989). Drought also promotes root and nodule senescence, facilitating the exudation of root exudates (Gogorcena et al., 1995; Mhadhbi et al., 2011). The abundance of exudates in the bulk soil and rhizosphere promotes the proliferation of microbes, which consequently improves enzyme activity. Aside from microbial-cell-released enzyme, exudates and lysed root tissue also release enzyme into the soil (Harvey et al., 2002; Chroma et al., 2002; Gramss et al., 1999).

1.5.3.2. Defoliation on soil microbiome and enzyme activity

Following defoliation, there is a general preference for reallocating nutrient reserves into the above-ground part to sustain the regrowth of photosynthetic tissues (Xu et al., 2013). This starves the below-ground tissues and decreases labile C sources (Fierer et al., 2007). These conditions promote oligotrophic bacterial taxa, which thrive under low C availability. However, following severe defoliation intensity, the majority of the reserves are transferred to the below-ground tissues (Aranjuelo et al., 2015; Hamilton et al., 2008; Wilson et al., 2018). The surplus of nutrients in the roots is then released into the soil as defoliation also induces root and nodule senescence (Hamilton et al., 2008; Carrillo et al., 2011; Thilakarathna and Raizada, 2019). The increased soil available nutrients cause the shift in the enrichment of copiotrophic bacterial taxa, which prefers the abundance of C sources (Ma et al., 2018).

1.5. Research hypothesis

Drought and defoliation stress negatively affect forage legume growth, plant physiological parameters, nodulation, symbiotic nitrogen fixation, consequently affecting soil enzyme activities and microbial dynamics.

1.6. Research objectives

The main objective of these research projects is to understand the effects of drought and defoliation stress on forage legume growth, plant physiological responses, symbiotic nitrogen fixation, and their consequential influence on soil enzyme and microbial dynamics in forage legumes.

The specific objectives of this research are:

1. Evaluate the effects of drought stress (mild and severe) on plant physiological parameters, plant biomass, root phenotypic traits, nodulation, symbiotic nitrogen fixation, and changes in soil nitrogen availability, extracellular enzyme activities, and soil bacterial community in alfalfa and red clover.
2. Evaluate the effects of defoliation stress (mild and severe) on plant physiological parameters, plant biomass, root phenotypic traits, nodulation, symbiotic nitrogen fixation, and changes in soil nitrogen availability, extracellular enzyme activities, and soil bacterial community in alfalfa and red clover.

Chapter 2 - Effect of drought stress on symbiotic nitrogen fixation, soil nitrogen availability and soil microbial diversity in forage legumes

Abstract

Forage legumes form mutualistic interactions with specialized soil rhizobia bacteria that inhabit root nodules and fix atmospheric nitrogen. However, legumes are sensitive to drought stress, which can interrupt nodulation and symbiotic nitrogen fixation (SNF). We hypothesize that drought-impaired SNF may influence soil nitrogen availability and soil microbial diversity. Here, we evaluated the effects of drought on nodulation, plant growth, physiological parameters, SNF, soil nitrogen availability, soil extracellular enzyme activity, and soil microbiome of alfalfa (*Medicago sativa*) and red clover (*Trifolium pratense*). The drought treatments were imposed at the flowering stage by maintaining soil moisture contents at 20% field capacity (FC) (severe drought), 40% FC (moderate drought), and 80% FC (well-watered) for three weeks. Drought significantly reduced nodulation, root and shoot growth, and SNF in alfalfa and red clover. Soil available nitrogen was significantly increased following severe drought conditions. The enzyme assays showed reduced activity of N-acetyl-glucosaminidase and β-D cellobiosidase enzymes under drought stress in alfalfa and red clover, respectively. Microbiome data showed shifts in the relative abundance of some key bacterial taxa under drought stress. Overall results indicate that drought has deleterious effects on SNF and plant growth, affecting carbon and nitrogen cycling enzymes, soil nitrogen availability, and soil microbial diversity.

Keywords: drought, forage legumes, symbiotic nitrogen fixation, soil enzymes, available soil nitrogen, soil microbiome

2.1 Introduction

Legumes play a crucial role in agriculture by providing protein-rich forage to livestock and improving soil health as a result of their symbiotic relationship with soil Rhizobia that allows them to fix nitrogen (Rochon et al. 2004; Rubiales and Mikic 2015). This legume-rhizobia relationship allows symbiotic nitrogen fixation (SNF), whereby atmospheric N_2 is reduced into bio-available nitrogen (i.e., ammonium) by catalysis of the nitrogenase enzyme present in rhizobial bacteria (Udvardi and Poole 2013). Alfalfa (*Medicago sativa*) and red clover (*Trifolium pratense*) are

among the top forage legumes for their high forage production, palatability, and nitrogen fixation, being able to fix ~ 78 to 222 kg N ha^{-1} yr^{-1} (Fageria 2014; De Haan et al. 2017). However, forage production and quality depend on adequate soil moisture availability (Hopkins and Prado 2007) and are often accompanied and influenced by various abiotic stresses (Zahran 1999; Laranjo et al. 2014). Insufficient soil moisture can strongly influence crop growth and biochemical and physiological processes (Enebe and Babalola 2018), decreasing forage yields and quality (Farooq et al. 2009; Jaleel et al. 2009). A critical challenge in this context is the identification of underlying mechanisms which limit legume production and SNF under drought conditions and the resulting impact on soil nitrogen availability and biological parameters.

Drought is one of the most crucial problems that hinder agricultural productivity (Lesk et al. 2016) and it is increasing in frequency and severity as a result of climate change (Schwalm et al. 2017). Therefore, there is a pressing need to focus research on improving the drought resistance of legumes to ensure global food security amidst climate change challenges. Drought induces several damaging effects on legume plants. Drought affects symbiosis initiation, where water deficit inhibits exchange of signaling molecules involved in host legume-rhizobia communication, resulting in poor nodulation and nitrogen fixation (Miransari et al. 2013). Water deficit not only reduces the abundance of established and introduced soil rhizobia in the soil but also their capacity for successful root infection, substantially reducing nodulation (Thilakarathna and Raizada 2017). Inhibition of nodule nitrogen fixation under drought stress is linked with carbon (C) shortage for rhizobia (Zargar et al. 2017), reduced nitrogenase activity due to oxygen limitation (del Castillo et al. 1994; Nishida and Suzaki 2018), and feedback inhibition of nitrogen fixation (Serraj et al. 2001; Valentine et al. 2011). Furthermore, drought stress induces cell dehydration, disrupting cell expansion and division, affecting leaf size, reducing stem elongation and root proliferation, impeding stomatal oscillations, plant water and nutrient uptake, and water-use efficiency (Kaushal and Wani 2016). The most common drought-induced plant responses are stomatal closure and reduction in photosynthesis rate (Liu et al. 2005; Mak et al. 2014). Moreover, water stress reduces the relative leaf expansion rate, affecting stomatal conductance and leaf turgor (Liu et al. 2005). Plants respond to drought by growing longer roots to reach deeper into the soil to access water (Kaspar et al. 1984), thus enhancing biomass partitioning to the roots and increasing root:shoot ratio (Manavalan et al. 2009).

Since plants are intimately interwoven with microbes living in and around the root system (Berg et al. 2016), plant physiology disruptions due to drought stress are expected to alter the plant microbiome and consequently, plant growth and health (Berg et al. 2014). Different plant species have varying rhizodeposition patterns (Cavaglieri et al. 2009), root architecture, and rooting depth, all of which have been shown to affect the structure of microbial communities (Chaparro et al. 2013, 2014; Fierer et al. 2003). Changes in root architecture, root growth, nodulation, and SNF in response to drought affect the rhizodeposits released into the soil (Preece and Peñuelas 2016; Jones et al. 2009), which will then affect the microbiome in the plant-soil interface (rhizosphere). Root exudates induce the interaction between the roots, the soil microbes and the surrounding soil particles. With the input of low-molecular-weight organic substances (Burns 1982; Dennis et al. 2010), soil microbes enriched by plant exudates play a critical role in diverse processes, including soil organic matter decomposition and nutrient cycling, by releasing and influencing various enzyme activities (Trasar-Cepeda et al. 2008; Kabiri et al. 2016; Bogati and Walczak 2022).

Previous studies have been conducted to leverage microbial communities for sustainable food production (De Vries and Wallenstein 2017; De Vries et al. 2020), and recent evidence shows that plant-associated microbes can play a role in alleviating drought stress (Lau et al. 2012; Niu et al. 2018). The effects of drought stress on above-ground plant traits have also been well-studied. Investigating the interdependent mechanisms of above and below ground plant traits, including interactions with microbes and relevant feedback, is crucial to developing a sustainable crop management strategy considering predicted climate change. This could involve exploring solutions, such as legume-based crop rotations, plant breeding, or using rhizobia inoculants.

Drought is well known to reduce legume productivity (Naya et al. 2007; Kang et al. 2011; Rouached et al. 2013), but mechanistic understanding of how drought impaired physiological and SNF parameters influences legume growth and its implications on soil health is lacking. Limited research has been conducted to evaluate and correlate the effects of drought stress on both above- and below-ground plant-growth parameters and the effect of drought-impaired SNF on soil nitrogen availability and soil microbial community. This study aimed to correlate the effects of moderate and severe drought stress on alfalfa and red clover growth and physiological response on legume SNF, nitrogen rhizodeposition, and its consequential influence on soil microbial and enzyme dynamics at the flowering stage. Specifically, we aimed to evaluate the effects of drought stress at the flowering stage on plant physiology, plant biomass (root and shoot), root phenotypic

traits, nodulation, nitrogen fixation, and changes in soil nitrogen availability, soil microbiome, and extracellular enzyme activities.

2.2 Materials and Methods

2.2.1 Plant growth conditions, rhizobia inoculation, and drought treatments

Alfalfa (OAC-Minto) and red clover (Juliet Double Cut) were selected for this study based on their plant vigour (root and shoot growth), persistence (Christie and Bennett 1984), and widespread use and being representative of typical legume ecology. Seeds were surface-sterilized by soaking in 70% ethanol for 2 min, then in 2% NaOCl solution for 3 min and rinsed with six changes of distilled water. Seeds were then pregerminated on sterile moistened filter paper in the dark at 28 ºC for two days (Thilakarathna and Raizada 2018). Seedlings were transplanted into 6.5 L pots (21.7 cm height, 22 cm diameter) with low-density polyethylene (LPDE) plastic lining that were filled with a 1:2 field soil (Black Chernozem soil) and sand (QUIKRETE® Premium Play Sand, QUIKRETE, Atlanta, GA, USA) potting mixture, mixed using a soil mixer for 10 min. Field soils were collected from the wheat stubble from the University of Alberta South Campus farm, where large particles were removed using a 0.6 × 0.6 cm size mesh. Final soil mixture had a total N content of 7.36 mg/kg. Five pre-germinated seeds were transplanted into each pot. Alfalfa and red clover seedlings were inoculated with *Sinorhizobium meliloti* 1021 and *Rhizobium leguminosarum* biovar *trifolii* Mj43, respectively (Thilakarathna and Raizada 2019). Each plant was inoculated with 1 ml of the inoculum, where rhizobia cell density was adjusted to OD600 = 0.1. After one week of growth, extra plants were removed, leaving three plants per pot. To measure SNF, two weeks and three weeks after the rhizobia inoculation, each plant was labelled with 25 ml of 0.5 mM $K^{15}NO_3$ solution (10 atom% ^{15}N; 348481-25G; Sigma Aldrich, Oakville, ON, Canada). Plants were supplied with 50 ml of quarter-strength N-free Hoagland's nutrient solution twice per week (pH = 6.8, adjusted using KOH) (HOP03-50LT, Caisson Labs, UT). Throughout the experiment, plants were kept in a greenhouse maintained at at 21 ± 2 °C with a 16/8 h light/dark photoperiod. Uninoculated legumes were grown in sterile sand-soil mix, labeled with ^{15}N-fertilizer similar to the plants inoculated with rhizobia.

Prior to conducting the experiments, the field capacity (FC) of the potting mixture was determined as described by Liyanage et al. 2022. Plants were introduced to drought treatments at the flowering stage, exposing them to 20% FC (severe drought), 40% FC (moderate drought), and

80% FC (well-watered) for three weeks. The specific moisture levels of the pots were achieved by weighing individual pots every other day and adding water to specific FC levels. Pots were arranged on the greenhouse bench in a randomized complete block design manner with five replicates per drought treatment (n = 5).

2.2.2 Plant physiological parameters

Following the three-week of drought period, the leaf chlorophyll content, linear electron flow (LEF), quantum yield (ΦII), and non-photochemical quenching (NPQ) were measured on fully expanded young leaves using a MultispeQ handheld unit (PHOTOSYNQ Inc. MI, USA). Three readings were taken per plant from different leaves of same maturity in a single day. All the readings were measured between 1:30 pm – 2:30 pm and data were synchronically saved in the PhotosynQ desktop software.

2.2.3 Measurement of soil available nitrogen

Following the three-week of drought treatments, three Plant Root Simulator probe pairs (anion and cation probes) (PRS™; Western Ag Innovations, Saskatoon, SK, Canada) were inserted into the soil around the plants. Soil moisture was brought back to 80% FC by applying water to facilitate NH_4^+ and NO_3^- diffusion into the probe surface. After one week of burial period under 80% FC, PRS probes were retrieved and immediately rinsed with a milli-Q water to remove any soil particles attached to the probes. The probes were packaged in Ziploc bags on ice and sent to the Western Ag Innovations laboratory (Saskatoon, SK) for analysis.

As for the analysis, PRS probe pairs were required to be eluted in Ziploc® bags with 17.5 mL of 0.5 M HCl solution for 1-h to ensure that ≥ 95% of adsorbed ions are obtained for elemental determination. Blank probes were also analyzed to ensure no N contamination/residual on the probe membranes. Soil nitrogen-supply rates were expressed as mg N/10 cm^2/burial period (10 cm^2 was the surface area of exchangeable resin) (Johnson et al. 2007).

2.2.4 Sampling of root nodules, roots and shoots

All the plants were harvested after retrieval of PRS probes. Plants were separated into shoots and roots. The root system of each plant was carefully uprooted from the soil and was briefly shaken to remove excess soil. Prior to root washing, 5–6 lateral root samples (each

measuring about ~ 12 cm) were clipped from each root system to serve as rhizosphere samples and were immediately kept at 4 ºC in tubes containing 35 ml of phosphate buffer. The remaining root systems were carefully washed free of soil and stored in the fridge in Ziploc bags. Roots were scanned using an Epson Expression 1640 scanner (Epson Canada Ltd.), and root architecture analysis was conducted using WinRHIZO software (Regent Instruments Inc.), which includes root length, volume, and surface area (Thilakarathna et al. 2016). Root nodules were removed from the root system and counted as single and cluster nodules. Dry weight of the nodules, shoots, and roots were measured after drying them in a 60 ºC oven for three days. The relative water content (RWC) of the shoot samples was calculated using the following formula based on fresh and dry weights (Basak et al. 2020):

$$RWC = ((Fresh\ weight - Dry\ weight)/Fresh\ weight) \times 100\%$$

2.2.5 Sampling of bulk and rhizosphere soil

Bulk soil samples were collected prior to the one-week recovery period, where plants remained at different moisture treatments. 2.5 cm diameter soil cores were collected using a metal corer at 12 cm depth and kept in Ziplock bags placed at -20 ºC. Rhizosphere samples were prepared following the protocol outlined by (McPherson et al. 2018). The tubes with root clippings and buffer were vortexed for 2 min. Roots were removed using sterilized forceps, and the resulting suspensions were centrifuged at 3000 g for 5 min at room temperature. The supernatant was discarded, and pellets were resuspended with 1.5 ml of phosphate buffer and vortexed briefly. Approximately 2 ml of suspension was transferred into new 2 ml microfuge tubes and centrifuged at 16,000 g for 2 min at room temperature. The supernatant was discarded, and pellets were stored at − 20 ºC.

2.2.6 Extracellular enzymatic activity (EEA) assay

An extracellular enzymatic activity (EEA) assay protocol was adapted from (Saiya-Cork et al. 2002) with modifications from the methods described by (German et al. 2011). Methylumbelliferone (MUB)-linked substrates were used to determine the activity of extracellular enzymes. One part of each soil sample was weighed for initial weight with moisture and then oven-dried at 75 ºC for two days for dry weight measurements. The other portion of the soil was used to obtain representative samples for the enzyme activity assays. Sample suspensions were prepared

by adding 2 g of soil to 125 ml of 50 mM acetate ($C_2H_3NaO_2$; CAS:127-09-3; BP333-500; Fisher Scientific; Toronto, ON). The pH of the acetate buffer was adjusted to 7.4 before adding samples. The solutions were homogenized for 10 min using a magnetic stirrer. The resulting suspensions were continuously stirred using magnetic stirrers while 200 µl of aliquots were dispensed into 96-well microplates.

Four enzymes critical for the cycling of soil organic C, N and P nutrients, were assayed in this study. Soil samples were buffered to their environmental pH (7.4) with 50 mM sodium acetate buffer solution. The assay was facilitated using a ratio of 2 g of soil per the standard 200 µM substrate concentration (Saiya-Cork et al. 2002). A standard hydrolytic enzyme assay using black Costar 96-well plates was used to quantify the activity of the soil extracellular enzymes (Saiya-Cork et al. 2002). Soil samples were incubated for 5 h at room temperature and were read using a Spectramax M3 plate reader (Molecular Devices, LLC. San Jose, CA, USA) at 365 nm excitation and 450 nm emission. Activities were calculated using standard equations on a per gram dry soil basis outlined by (German et al. 2011). Specific enzymes assayed are as follows: (1) cellulolytic enzymes β-1, 4-glucosidase (BG) and β-D-cellobiosidase (Cello) responsible for release of glucose for microbial C acquisition; (2) phosphatase enzyme phosphatase (Phos) responsible for the release of inorganic P; and (3) glucosaminidase enzyme N-acetyl-β glucosaminidase (NAG) which catalyzes the breakdown of organic matter (e.g. chitin fungal cell wall) releasing nitrogen compounds (Hewins et al. 2015).

2.2.7 Measurement of symbiotic nitrogen fixation

Dried shoot materials were ground using a SPEX SamplePrep 8000 M Mixer/Mill ball grinder for 10 min, followed by Beadruptor (Beadruptor 12 Homogenizer, Omni International Inc.) to turn the sample into fine powder. In order to measure ^{15}N and total N concentration of the shoot samples, 5 mg of ground sample was measured and transferred into small tin capsules (8 mm × 5 mm, D1008, standard weight, Isomass Scientific Inc.) using a microbalance (Liyanage et al. 2023). The capsules were arranged in a 96-well plate and sent to the Stable Isotope Facility at Agriculture and Agri-Food Canada, Lethbridge, for ^{15}N isotope analysis. Samples were analyzed for ^{15}N and total N% using a Finnigan Delta V Plus (Thermo Electron) Isotope Ratio Mass Spectrometer (IRMS) with a Flash 2000 Elemental Analyzer (Thermo Fisher Scientific). The percentage

nitrogen derived from the atmosphere (%Ndfa) of the shoot samples was calculated using the following formula according to the isotope dilution technique (Liyanage et al. 2023):

$$\%Ndfa = \left(1 - \frac{\text{atom\% } {}^{15}N \; excess_{(fixing\ plant)}}{\text{atom\% } {}^{15}N \; excess_{(non-fixing\ plant)}}\right) \times 100$$

Alfalfa and red clover plants that were not inoculated with rhizobia (hence not -nodulated) from the same experiments were used as the references.

2.2.8 Bacterial DNA extraction, sequencing, and bioinformatics analyses

Total DNA from bulk soil and rhizosphere soil samples were extracted using the Qiagen DNeasy PowerSoil Pro kit (Qiagen, Germany) following the manufacturer's protocol. Genomic DNA concentration and purity were measured using a NanoDrop 2000 (NanoDrop Technologies, Wilmington, DE) spectrophotometer. DNA samples were then sent to Laval Genomics Platform (University of Laval, Quebec, Canada) for Illumina Miseq paired-end sequencing of the bacterial 16S rRNA V3-V4 region using primers — 341F 3'-GCCTACGGGNGGCWGCAG-5' and 806R 3'-ACTACHVGGGTATCTAATCC-5'. The amplicon libraries were prepared using a Nextera XT index kit (Illumina Inc. USA) following metagenomic sequencing library preparation protocol. The paired-end raw reads were then quality filtered and contigs created using the Mothur pipeline (v.1.42) (Schloss et al. 2009). Chimeric sequences were removed using VSEARCH (Rognes et al. 2016). Sequences were aligned and OTUs clustered using the RDP classifier database (Cole et al. 2014). MicrobiomeAnalysts (Chong et al. 2020) was used for the succeeding bioinformatics analysis consisting of alpha and beta diversity, and relative abundance. To address the variability in sequencing depth among samples, sequences were rarified to the minimum library size (Weiss et al. 2017) implemented in MicrobiomeAnalysts and were used in all downstream analyses. The raw sequence data in this study were deposited in the NCBI Sequence Read Archive under the accession number PRJNA1012008.

2.2.9 Data Analyses

All statistical analyses were performed using R v.4.2.3 (R core Team 2023). The effect of different moisture treatments on nodulation, photosynthetic parameters, plant biomass, root

parameters, relative leaf chlorophyll, shoot N, C/N ratio, carbon isotope discrimination (CID), SNF, soil available N, soil enzyme activities, and microbial diversity and taxonomic abundances were analyzed using ANOVA set at $p < 0.05$ using the "aov" function with assumptions that the responses were from normal a population distribution (Shapiro-Wilk test function "shapiro.test" in base R) with equal variances (Levene's test function "leveneTest" from car package v.3.1-2). Significant differences among treatments were determined by the Tukey test ("TukeyHSD" function in base R). Where assumptions of normality and homogeneity of variance were not met, the non-parametric Kruskal-Wallis test ("kruskal.test" function from base R) was used, followed by Dunn's test) to determine significant differences (Pohlert 2022). We used principal component analysis (PCA) as an exploratory data analysis to visualize multivariate responses to drought treatments. PCA figures were generated using the autoplot function in ggplot2 v.3.3.6 with loading.label.repel formatting from ggfortify v.0.4.16.

Both alpha- and beta-diversities were analyzed in the MicrobiomeAnalyst (Chong et al. 2020) implementation. Permutational multivariate analysis of variance (PERMANOVA) using Bray-Curtis dissimilarity was done to identify significant differences in bacterial community structure impacted by drought exposure. In MicrobiomeAnalyst implementation, the p-values were corrected for multiple testing using the Benjamini and Hochberg's False-Discovery Rate (FDR).

2.3 Results

2.3.1 Nodulation

Nodulation was measured to evaluate the effect of drought on number of nodules and nodule dry weight. Compared to the well-watered control, the number of single nodules was significantly reduced at 40% FC and 20% FC in alfalfa ($P < 0.01$; Fig. 2.1A), while only significantly reduced under 20% FC in red clover ($P < 0.01$; Fig. 2.1B). In alfalfa, the number of nodule clusters was significantly reduced only under severe drought (20% FC) compared to the 80% FC treatment ($P < 0.05$; Fig. 2.1C). However, the number of cluster nodules was not significantly different under drought treatments in red clover compared to the well-watered treatment (Fig. 2.1D). Comparison to the well-watered control, the total number of nodules per plant (single nodules and cluster nodules) was significantly reduced under both drought stress levels in alfalfa plants ($P < 0.01$; Fig. 2.1E). In contrast, there was only a significant reduction in total nodule number under severe drought conditions in red clover ($P < 0.01$; Fig. 2.1F). Lastly,

the nodule dry weight was not significantly different among different soil moisture treatments in alfalfa (Fig. 2.1G), but was significantly reduced in red clover at 20% FC compared to the 80% FC control ($P < 0.01$; Fig. 2.1H).

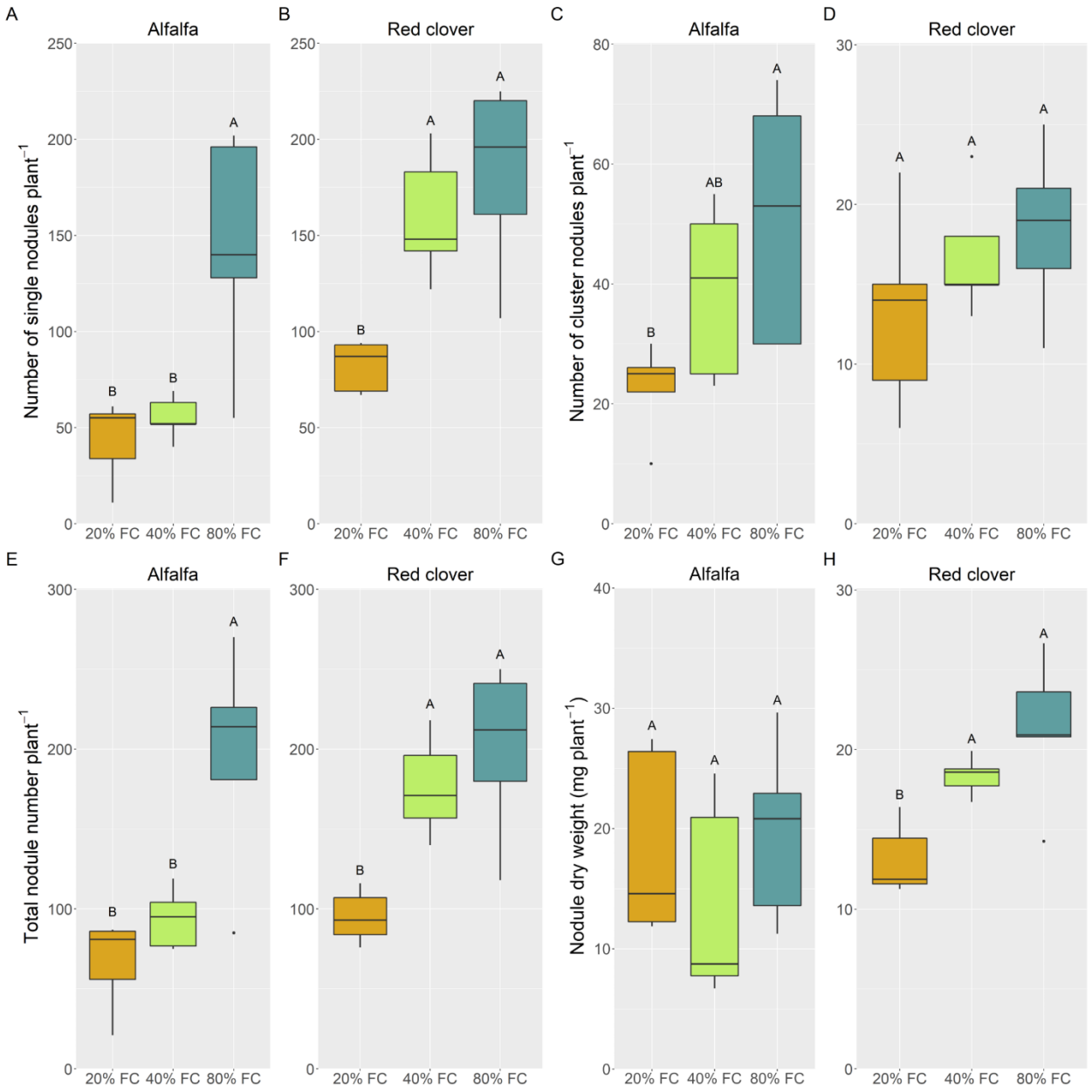


Figure 2.1 The effects of three weeks of moderate (40% field capacity) and severe (20% field capacity) drought stress on nodulation parameters in alfalfa and red clover compared to the well-watered control (80% field capacity). A-B Number of single nodules per plant; C-D Number of cluster nodules per plant; E-F Total nodule number per plant; G-H Nodule dry weight per plant.

FC, Field capacity. Different letters indicate significant differences between soil moisture treatments at $p < 0.05$. Each treatment comprised five replicate pots.

2.3.2 Plant physiological parameters

Plant physiological parameters were measured to understand the responses of alfalfa and red clover to drought stress. The relative leaf chlorophyll content (measured as SPAD units) in alfalfa plants was reduced under 20% FC drought treatment (20.4 SPAD) compared to 40% FC drought (40.8 SPAD, $P < 0.01$) (Fig. 2.2A). Conversely, in red clover, leaf chlorophyll content was significantly higher under 20% FC (52.4 SPAD) drought treatments compared to the 80% FC well-watered plants (35.8 SPAD; $P < 0.05$), whereas no significant difference was observed in 40% FC (49.2 SPAD) compared to well-watered and severe drought treatments (Fig. 2.2B). Following three weeks of drought treatments, there were no significant differences in the LEF of the plants in response to drought (Fig. 2.2C, D). Quantum yield is the light energy being transported into Photosystem II (ΦII) to be actively converted into usable energy. In alfalfa, the quantum yield measured using the MultiSpeQ was not significantly different among the three moisture levels (Fig. 2.2E), while there was an increased ΦII in moderate drought compared to well-watered in red clover ($P < 0.05$; Fig. 2.2F). On the other hand, non-photochemical quenching is a defense response of plants to dissipate excess light energy, that is as heat, to prevent damage. The measured non-photochemical quenching in the alfalfa was significantly higher in plants under 20% FC drought treatment compared to 80% FC control plants ($P < 0.05$; Fig. 2.2G), while there were no significant differences among the three moisture treatments in red clover (Fig. 2.2H).

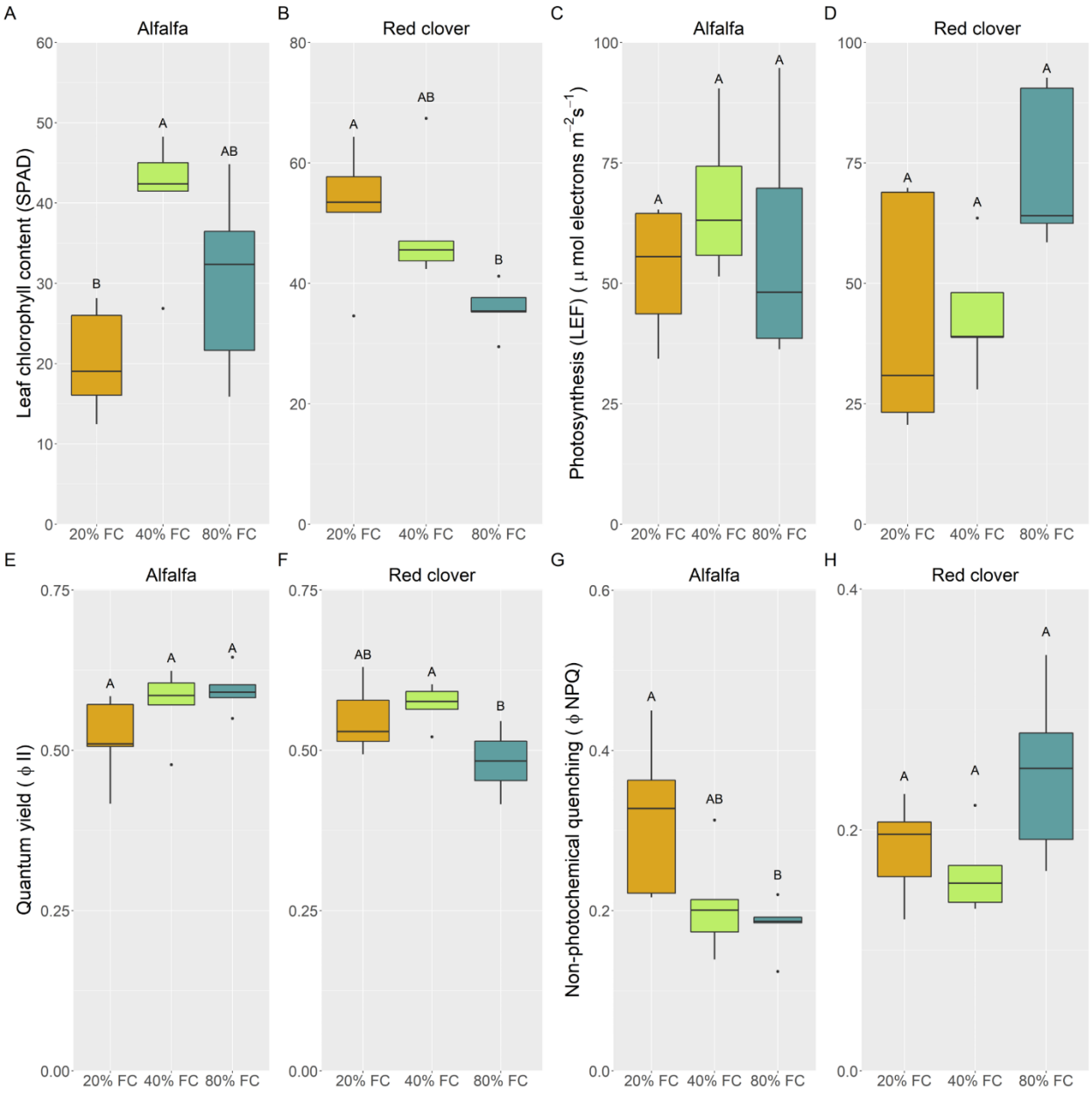


Figure 2.2 The effects of three weeks of moderate (40% field capacity) and severe drought (20% field capacity) stress on plant physiological parameters in alfalfa and red clover compared to the well-watered control (80% field capacity). A-B Leaf chlorophyl content; C-D Leaf photosynthesis (LEF); E-F Quantum yield (ΦII); G-H Non-photochemical Quenching (ΦNPQ). FC, Field capacity; Different letters indicate significant differences between soil moisture treatments at $p < 0.05$. Each treatment comprised five replicate pots.

2.3.3 Plant biomass and root phenotypic traits

We measured the shoot and root growth and root architecture parameters under drought stress to understand how plant growth parameters related to drought impaired SNF. In alfalfa, the shoot dry weight was reduced by 36% under moderate drought (40% FC) and 59% under severe drought (20% FC) compared to the well-watered plants (80% FC) ($P < 0.01$; Fig. 2.3A). Similarly, shoot dry weight in red clover was also reduced by 38% under moderate drought and 64% under severe drought compared to the well-watered control ($P < 0.001$; Fig. 2.3B). The RWC in alfalfa shoots was slightly higher in well-watered plants (73.3%) compared to the plants exposed to moderate (70.0%) and severe drought (70.1%) (Fig. A.1A). In contrast, there was significantly lower shoot RWC in red clover plants exposed to moderate (54.4%) and severe drought (34.3%) compared to the well-watered plants (64.3%) (Fig. A.1B). Concerning root dry weight, alfalfa plants were significantly reduced under severe drought stress ($P < 0.01$; Fig. 2.3C) while both moderate and severe drought significantly reduced the root dry weight in red clover by 41% and 50%, respectively ($P < 0.0001$; Fig. 2.3D). Although there was a declining trend for total root length under drought stress, root length was not significantly different between drought treatments and 80% FC well-watered control in alfalfa and red clover (Fig. 2.3E, F). Root surface area was not significantly reduced in alfalfa plants under 20% and 40% FC treatment compared to well-watered treatment, possibly due to high variability between replicates (Fig. 2.3G). On the other hand, the root surface area was significantly lower in red clover plants under 40% FC and 20% FC compared to the well-watered plants ($P < 0.05$; Fig. 2.3H). Although different soil moisture treatments had no significant impact on root volume in red clover, 20% FC severe drought significantly reduced the root volume in alfalfa compared to the well-watered control (Fig. 2.3I). The average root diameter was significantly reduced only in red clover under 40% FC and 20% FC compared to the 80% FC ($P < 0.0001$; Fig. 2.3L).

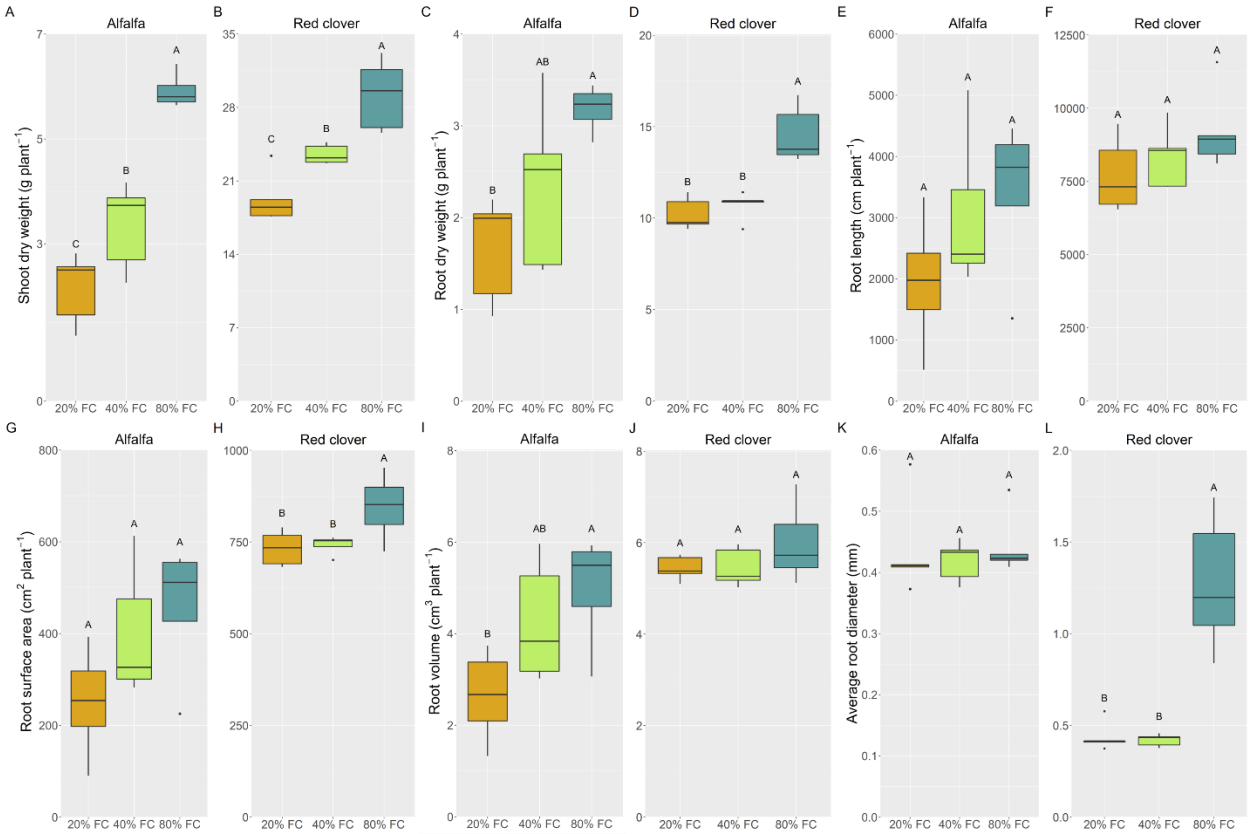


Figure 2.3 The effects of three weeks of moderate (40% field capacity) and severe drought stress (20% field capacity) on plant biomass and root traits of alfalfa and red clover compared to the well-watered control (80% field capacity). A-B Shoot dry weight; C-D Root dry weight; E-F Total root length; G-H Root surface area; I-J Root volume; K-L Average root diameter. FC, Field capacity; Different letters indicate significant differences between soil moisture treatments at $p < 0.05$. Each treatment comprised five replicates pots.

2.3.4 Effect of drought stress on shoot nitrogen, carbon isotope discrimination, and nitrogen fixation

Alfalfa plants exposed to severe drought (20% FC) had a lower shoot N concentration compared to the well-watered plants (80% FC) (Fig. 2.4A). However, no changes in shoot N concentration were observed in red clover plants subjected to different soil moisture levels (Fig. 2.4B). A similar pattern was observed for shoot total N content, where both drought treatments significantly reduced the shoot total N content in alfalfa ($P < 0.01$; Fig. 2.4C), while there was no effect on red clover ($P < 0.01$; Fig. 2.4D). The shoot C/N ratio was determined based on the C and

N concentrations of the shoot samples. Alfalfa grown under the severe drought condition had a higher shoot C/N ratio compared to moderate and well-watered conditions ($P < 0.001$; Fig. 2.4E). In contrast, red clovers grown under the moderate drought condition had a higher shoot C/N ratio compared to severe drought and well-watered control treatments although not statistically significant (Fig. 2.4F). The carbon isotope discrimination (CID) of the shoot sample was significantly lower in alfalfa under both drought treatments compared to the well-watered condition ($P < 0.01$; Fig. 2.4G) indicating higher water use efficiency under drought stress conditions compared to the well-watered conditions. However, there was no significant difference in CID in red clover plants grown under different moisture conditions (Fig. 2.4H). The N fixation capacity (%Ndfa) of alfalfa was not significantly different between drought and well-watered treatments (Fig. 2.4I). In contrast, %Ndfa was significantly lower in red clover plants subjected to severe drought conditions compared to well-watered conditions ($P < 0.01$; Fig. 2.4J). The total N fixation in alfalfa was significantly lower under both drought treatments compared to the well-watered condition ($P < 0.01$; Fig. 2.4K), wherein only the 20% FC severe drought significantly reduced the total N fixed in red clover ($P < 0.01$; Fig. 2.4L).

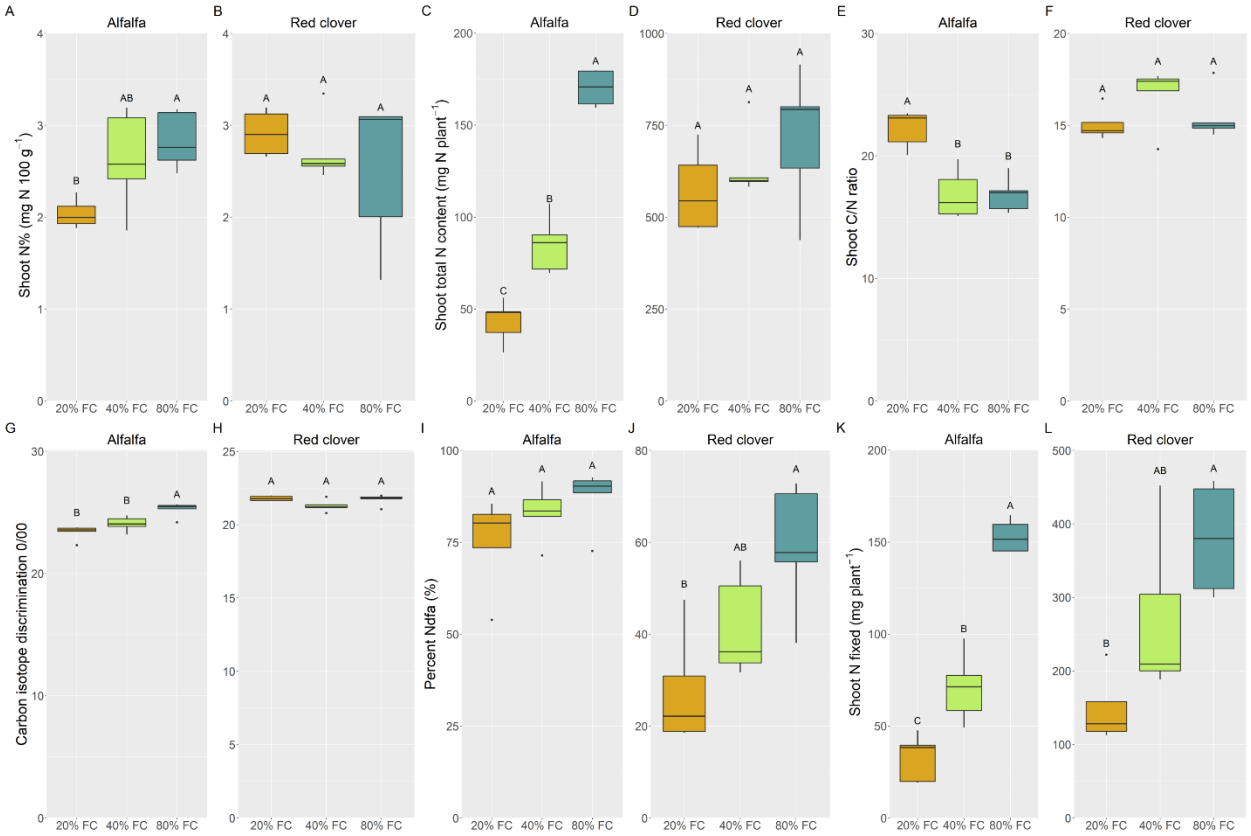


Figure 2.4 The effects of three weeks of moderate (40% field capacity) and severe drought stress (20% field capacity) on shoot nitrogen, carbon isotope discrimination, and nitrogen fixation compared to the well-watered control (80% field capacity). A-B Shoot nitrogen concentration; C-D Shoot total N content per plant; E-F Shoot C/N ratio; G-H Carbon isotope discrimination in shoots; I-J Percent (%) nitrogen derived from the atmosphere (%Ndfa); K-L Total shoot nitrogen fixed per plant. FC, Field capacity; Different letters indicate significant differences between soil moisture treatments at $p < 0.05$.

2.3.5 Soil available nitrogen and extracellular enzyme

Measurement of soil nitrogen and the extracellular enzyme activity is important to understand the effects of drought on N rhizodeposition and its influence on extracellular enzyme activity. The available soil nitrogen (NO_3^- and NH_4^+) was measured using plant root simulator (PRS) probes. The majority of the available nitrogen was in the form of NO_3^- in both crops across all the moisture treatments. PRS probes retrieved a minimal amount of NH_4^+ across all watering regimes resulting in no statistical differences among the treatments in alfalfa and red clover (Fig.

A.2). The available soil NO_3^- and total available soil N were higher under 20% FC severe drought conditions compared to the well-watered control ($P < 0.05$; Fig. 2.5A, D). Overall results showed that soil extracellular enzymes have a uniform response to drought effects on their activity, with one exception in each trial. First, β-1, 4-glucosidase (BG) and β-D-cellobiosidase (Cello), both of which are carbon cycling enzymes, were not influenced by drought conditions in alfalfa (Fig. 2.5E, G). Although the BG activity was similar across different moisture treatments in red clover, Cello was observed to have decreased activity under severe drought conditions compared to well-watered control ($P < 0.01$; Fig. 2.5H). Second, results showed that the phosphate hydrolyzing enzyme phosphatase (Phos) was not affected by drought stress in both crops (Fig. 2.5I, J). Lastly, the nitrogen liberating enzyme N-acetyl-β glucosaminidase (NAG) was substantially affected by drought treatments compared to well-watered conditions in alfalfa ($P < 0.01$; Fig. 2.5K), while no effects in NAG activity were observed under red clover (Fig. 2.5L).

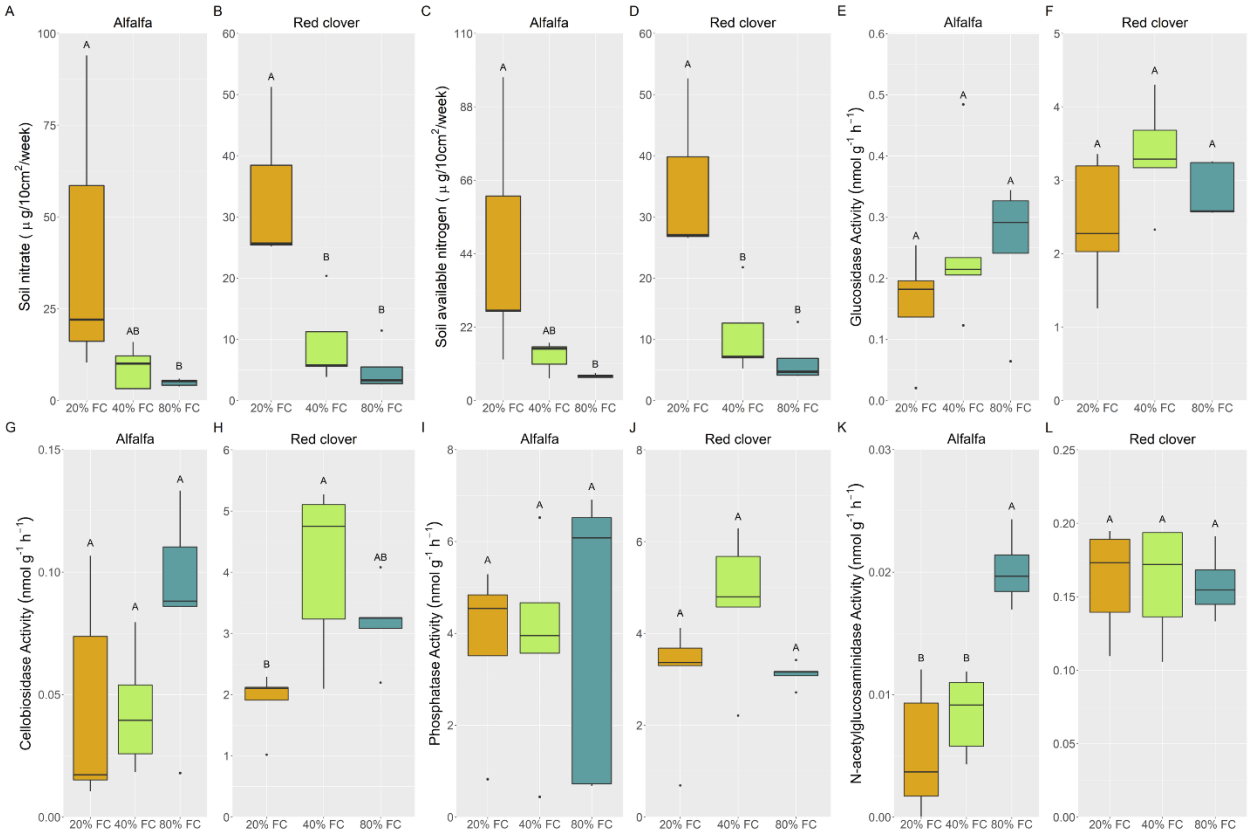
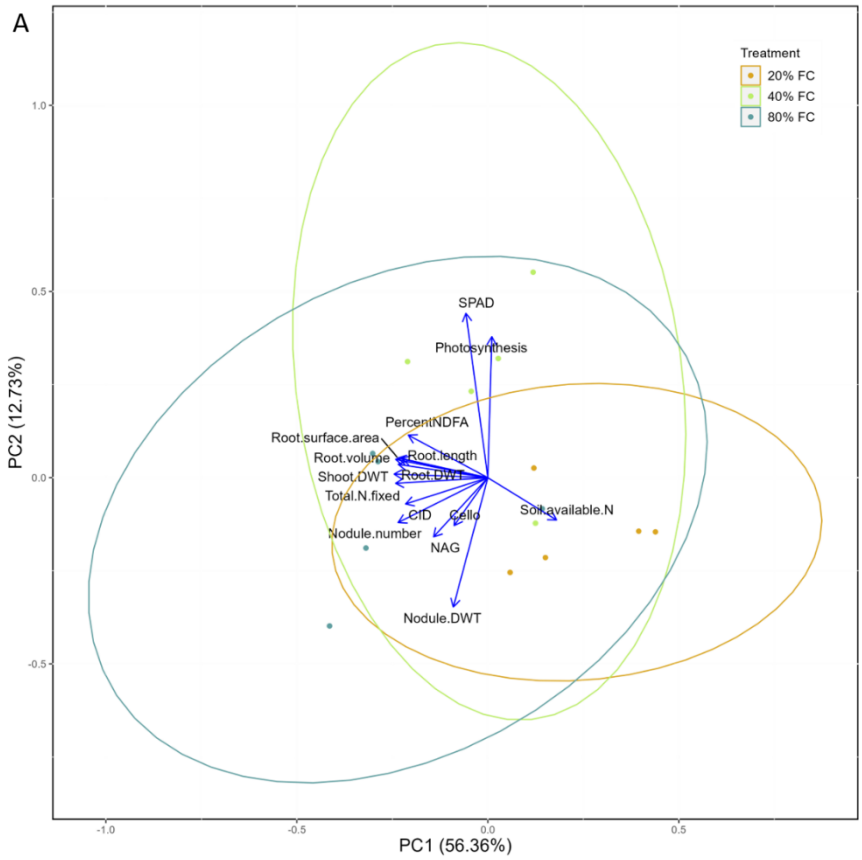


Figure 2.5 The effects of three weeks of moderate (40% field capacity) and severe drought stress (20% field capacity) on soil available nitrogen forms and soil carbon, nitrogen, and phosphorous

extracellular enzymes compared to the well-watered control (80% field capacity). A-B soil nitrate; C-D Total soil available nitrogen; E-F Glucosidase (Glu) activity; G-H Cellobiosidase (Cello) activity; (I-J) Phophatase (Phos) activity; K-L N-acetylglucosaminidase (NAG) activity. FC, Field capacity; Different letters indicate significant differences between soil moisture treatments at $p < 0.05$.

2.3.6 Principal component analysis of different plant and soil parameters

In the PCA biplot, the first two components accounted for 69% of total variance in the 15 attributes considered in alfalfa (Fig. 2.6A), while 64% was accounted in red clover (Fig. 2.6B). As observed, drought-treated individuals clustered together, distinctly separating the three moisture levels from each other. The loading variables in alfalfa that were positively correlated with well-watered condition ordination space were as follows: nodule number, nodule dry weight, shoot and root dry weight, root length, root volume, root surface area, CID, total nitrogen fixed, %NDFA, Cello, and NAG. Both SPAD and photosynthesis positively correlated with moderate drought conditions, while only total soil available nitrogen positively correlated with severe drought conditions. Furthermore, the loadings in red clover that positively correlated with well-watered were: nodule number, nodule dry weight, photosynthesis, root and shoot dry weight, root length, root volume, root surface area, %NDFA, CID, and total nitrogen fixed. Celliobiosidase was the only variable correlated positively with moderate drought, while SPAD and soil available nitrogen positively correlated with severe drought conditions in red clover.


A
Treatment
20% FC
40% FC
80% FC
SPAD
Photosynthesis
PercentNDFA
Root.surface.area
Root.volume
Root.length
Shoot.DWT
Root.DWT
Total.N.fixed
CID
Cello
Soil.available.N
Nodule.number
NAG
Nodule.DWT
PC2 (12.73%)
PC1 (56.36%)

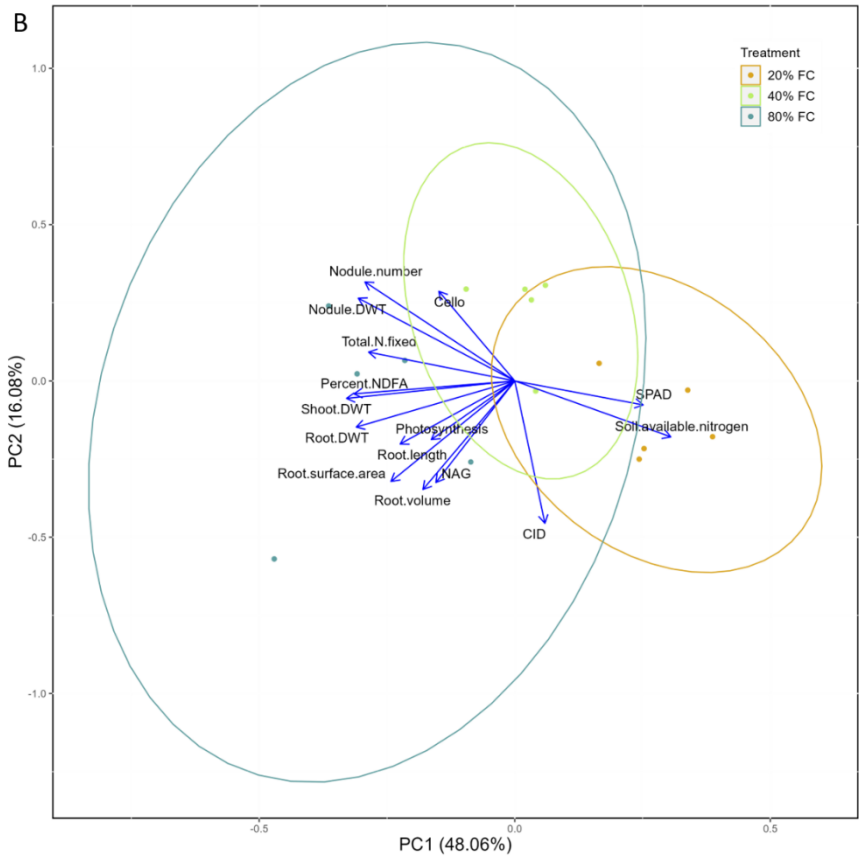

B
Treatment
20% FC
40% FC
80% FC
Nodule.number
Cello
Nodule.DWT
Total.N.fixed
Percent.NDFA
SPAD
Shoot.DWT
Soil.available.nitrogen
Photosynthesis
Root.DWT
Root.length
Root.surface.area
NAG
Root.volume
CID
PC2 (16.08%)
PC1 (48.06%)

Figure 2.6 Principal component analysis of nodulation parameters (nodule number, dry weight), physiological parameters (leaf chlorophyll content - SPAD, Photosynthesis), shoot and root biomass, root architecture (length, volume, surface area), root/shoot ratio, shoot N%, shoot C/N ratio, percent nitrogen derived from the atmosphere (%Ndfa), total N fixed, and carbon isotope discrimination (CID), soil available nitrogen, and extracellular enzyme activity (Cellobiosidase-Cello, N-acetylglucosaminidase-NAG) of A alfalfa and B red clover plants grown under well-watered (80% field capacity), moderate drought (40% field capacity), and severed drought (20% field capacity) conditions. DWT, dry weight.

2.3.7 Microbiome Analysis

We investigated the impact of drought on the soil and rhizosphere bacterial community structure of forage legumes. Actinobacteria predominated both plants' bulk and rhizosphere soil at the phylum level, followed by Proteobacteria¸ Firmicutes, Acidobacteria, and an unclassified phylum (Fig. 2.7A, B). Performing alpha-diversity analysis in bulk soil for both plants, Shannon diversity indices were higher in moderate drought than in severe and well-watered treatments (Fig. 2.8A, B), though not statistically significant. The drought impact on bacterial diversity, although not statistically significant, was more pronounced in the alfalfa rhizosphere showing a trend in lower Shannon diversity (Fig. 2.8C), as well as Chao1 and Observed indices (Fig. A.3), in drought treatments. In contrast, bacterial diversity in the red clover rhizosphere was not affected by drought (Fig. 2.8D). Furthermore, we did not observe a shift in the bacterial community in either plant due to drought. This was determined through permutational analysis of variance using the Bray-Curtis dissimilarity matrix (Kers and Saccenti 2021). For both plants, the bacterial community in the rhizosphere was clearly distinct from the bulk soil, reflecting the rhizosphere effect and specialist bacteria living in the root zone recruited by the host plant from the bulk soil ($P < 0.01$; Fig. A.4).

The impact of drought on the relative abundances of some taxonomic groups was more evident in alfalfa bulk soil (Fig. 2.8E-G). The relative abundances of Acidobacteria were lower in drought treatments; in particular, moderate was significantly lower than the well-watered ($P < 0.05$; Fig. 2.8E). Conversely, the relative abundances of phylum Proteobacteria ($P < 0.05$; Fig. 2.8F) and family Sphingomonadaceae ($P < 0.05$; Fig. 2.8G) under severe drought significantly increased compared to the well-watered treatments. We observed taxonomic enrichment in the

rhizosphere under drought on red clover only, as shown by the increase in the relative abundances of Nocardioidaceae ($P < 0.05$; Fig. 2.8H).

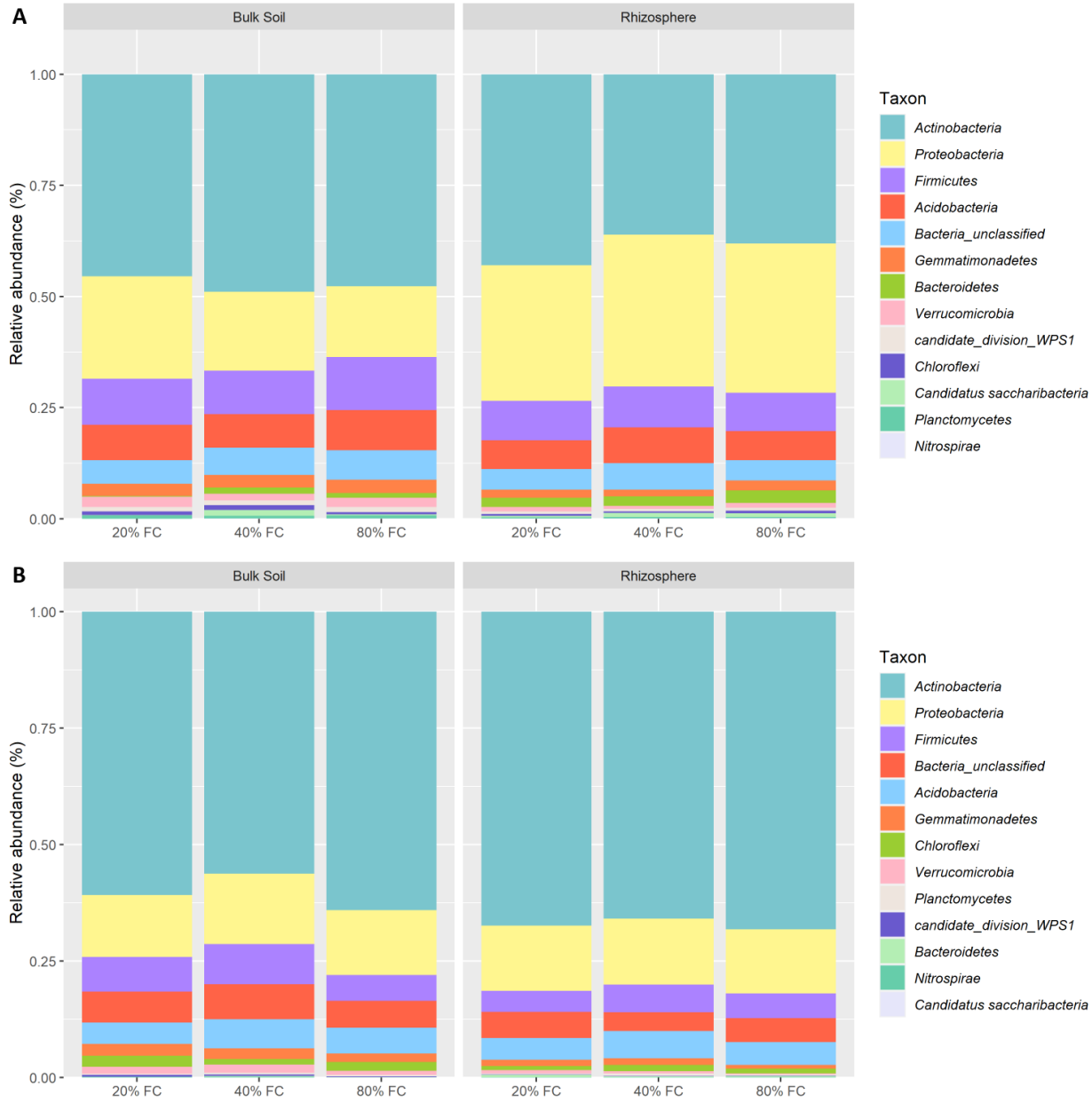


Figure 2.7 Distribution and abundance analysis of bacterial communities in bulk and rhizosphere soil under alfalfa and red clover under different soil moisture conditions. A Alfalfa phyla relative abundance; B Red clover phyla relative abundance. Legend shows phylum phylogenetic level.

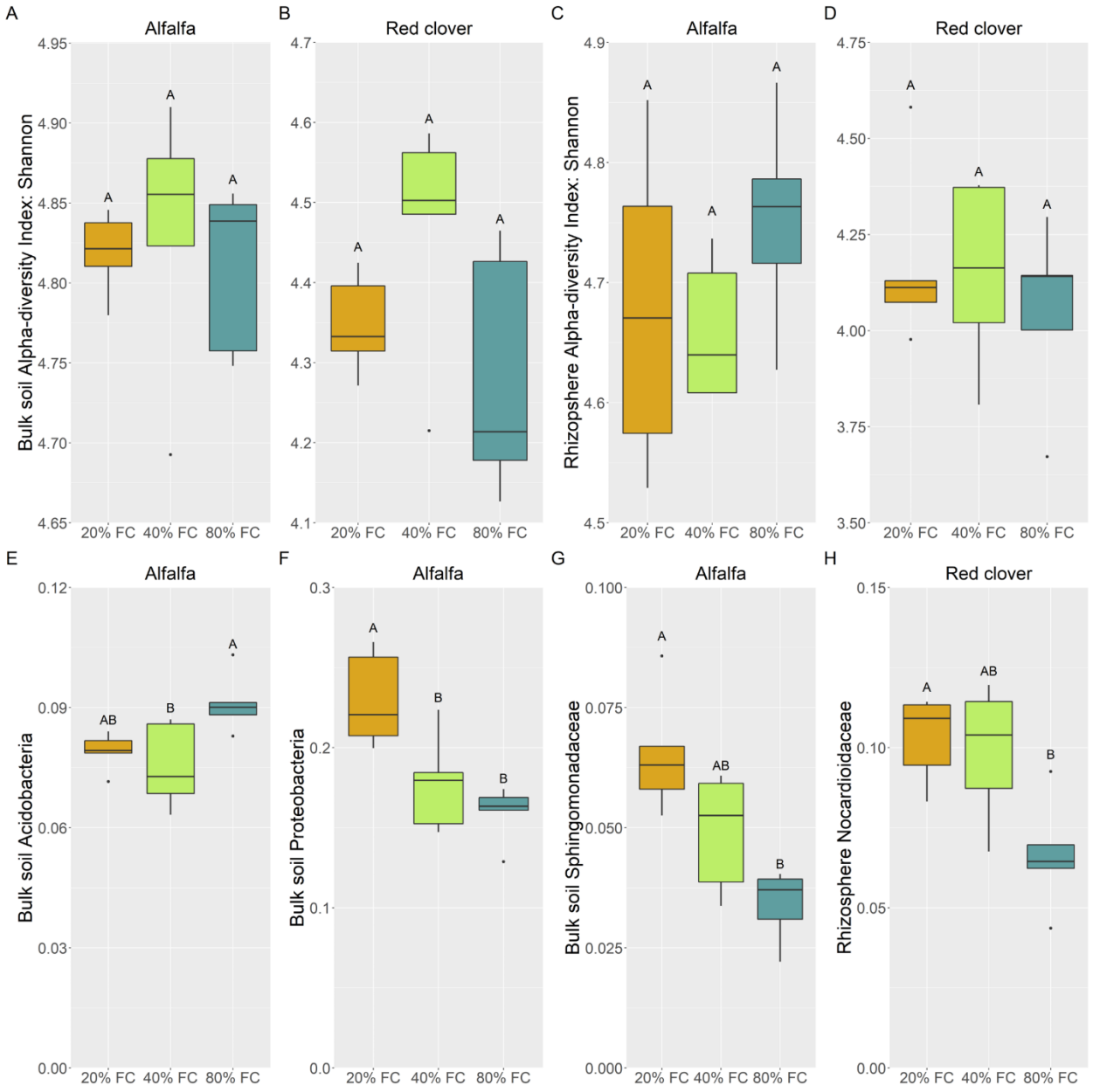


Figure 2.8 Microbiome analysis of alfalfa and red clover soil and rhizosphere samples under moderate (40% field capacity) and severe (20% field capacity) drought conditions, and well-watered (80% field capacity) as control. Shannon diversity indices of alfalfa and red clover bulk (A-B) and rhizosphere (C-D) samples. Relative abundances: Acidobacteria (E), Proteobacteria (F), and Sphingomonadaceae (G) in alfalfa bulk soil; Nocardioidaeae (H) was significantly enriched in the red clover rhizosphere. FC, Field capacity; Different letters indicate significant differences between soil moisture treatments at $p < 0.05$.

2.4 Discussion

Overall results indicates that drought has deleterious effects on nodulation, plant growth, and carbon and nitrogen cycling enzymes while positively impacting soil nitrogen availability and some specific soil microbial taxa. These significant changes in legume plant growth, nodulation, physiological parameters, and SNF and its consequential effects on soil enzymes and the microbe community help us to understand the overall response of legumes under drought stress. The greenhouse experiments described here were conducted to assess the effect of soil moisture limitation on two essential forage legumes, alfalfa and red clover, by using passive pot-drying by withholding irrigation at different field capacity levels (Poorter et al. 2012; Marchin et al. 2020). Faced with finite water resources, drought has been known as the most critical threat to global food security by constraining crop yield (Micheletto et al. 2007; Farooq et al. 2017). Furthermore, drought is predicted to increase in frequency and severity due to global climate change (Lesk et al. 2016; Dey et al. 2019). Therefore, evaluating how drought stress affects forage legume growth, physiological parameters, symbiotic nitrogen fixation, and its consequences on soil nitrogen availability and microbial diversity will help to understand the system responses.

Legume root nodulation is sensitive to drought stress, and this current study describes the impact of moderate and severe drought conditions on alfalfa and red clover nodulation. Overall results suggest that nodulation in forage legumes can be negatively affected under severe drought conditions. Previous studies with grain legumes also confirmed the reduction in nodulation under drought stress (Lumactud et al. 2023; Fernández-Luqueño et al. 2008; Marquez-Garcia et al. 2015). The success of root nodulation starts from a healthy rhizobia population size in the soil (Rehman and Nautiyal 2002), where prolonged drought conditions can reduce soil rhizobia population, thus reducing nodulation (Herrmann et al. 2014; Kasper et al. 2019). Drought can impair the legume-rhizobia signaling exchange, disrupting the establishment of symbiosis (Thilakarathna and Cope 2021; de Freitas et al. 2023). We observed varying specific responses of red clover and alfalfa to drought. Our results showed that the average nodule dry weight was significantly reduced under severe drought conditions in red clover but not in alfalfa. The ability of alfalfa plants to maintain their nodule mass while exhibiting a significant reduction in cluster nodules and total nodule number is possibly another adaptive mechanism under drought conditions (Lumactud et al. 2023). Specifically, alfalfa plants may have compensated by maintaining only the large nodules.

Leaf chlorophyll content (SPAD), linear electron flow (LEF) as proximate to photosynthesis, quantum yield (ΦII) and non-photochemical quenching (ΦNPQ) are the common photosynthetic parameters measured using the MultispeQ (Kuhlgert et al. 2016). In alfalfa, peas, and soybean, the rates of photosynthesis were reduced under short and prolonged (two to three months) drought stress by limiting the carbon influx via the stomata and reduction of chlorophyll content (Zargar et al. 2017; Zhang et al. 2018; Wang et al. 2018; Jacques et al. 2022). Our present study did not support these claims, as the photosynthesis was not significantly different among different FC treatments. A possible explanation for this contradictory result is that alfalfa and red clover may have higher drought tolerance for photosynthesis or have acclimated to drought over three weeks of the drought period. A determined long-term measurement linked to photosynthesis is the amassment of leaf chlorophyll. Interestingly, we found a significant increase in alfalfa leaf chlorophyll content under moderate drought stress compared to the well-watered control after three weeks of drought period. This increased leaf chlorophyll content may be linked to sustaining photosynthesis by increasing chlorophyll pigments. Furthermore, photosynthesis efficiency is described by ΦII and ΦNPQ. The ΦII, the energy conversion in photosystem II (PSII), has an inverse relation with ΦNPQ, which is the non-photochemical energy loss responsible for protecting PSII from photodamage (Parida et al. 2007). We report higher ΦNPQ results in lower ΦII, as shown in Fig. 2.2E-H. Similar results were reported by Mwale et al. (2017) in cowpea under drought stress.

Drought stress can still reduce biomass production (Mouradi et al. 2016; Begum et al. 2019), supporting our findings on the reduced shoot and root dry weight under 40% and 20% FC treatments. In our study, shoot biomass was reduced by 59% and 34% under severe drought stress in alfalfa and red clover, respectively, compared to the well-watered control plants. Similarly, root biomass was reduced by 40% and 30% under severe drought stress in alfalfa and red clover, respectively, compared to the well-watered control plants. Drought causes changes in cell metabolism (Vaseva et al. 2011), affecting cell elongation and enlargement, resulting in decreased growth (Ashraf and O'Leary 1996), which supports the significant changes in root architecture for both alfalfa and red clover (Fig. 2.3).

It is widely accepted that drought significantly affects SNF in legumes, resulting in the decline of shoot N content. Although we found a significant reduction in %Ndfa in red clover under severe drought stress conditions, we did not see the same trend in alfalfa. Several factors are

linked to the inhibition of N_2 fixation under drought. The decline in nodule sucrose synthase results in the shortage of carbon substrates needed to facilitate SNF (González et al. 2015; Kunert et al. 2016). Another factor is oxygen limitation, where oxygen diffusion is limited due to loss of turgor and cell volume, disrupting the microaerobic environment, resulting in the inhibition of nitrogenase activity (Serraj et al. 1999; Serraj 2003; González et al. 2015). Furthermore, drought conditions induce ABA synthesis, consequently reducing the synthesis of leghaemoglobin and dramatically increasing the oxygen diffusion resistance into the nodules (Naya et al. 2007; González et al. 2015). It is important to note that the ^{15}N dilution method used to measure SNF in this study provided time-integrated measurements for %Ndfa, in contrast to the acetylene reduction assay that provided instantaneous N_2 fixation values during the drought period (Thilakarathna et al. 2017). Additionally, soil sterilization in non-nitrogen fixing reference plants may have imposed an additional variable as there is a possibility that the soil microbes consumed a small portion of the applied ^{15}N fertilizer in the unsterilized soil mixture, which affects %Ndfa calculations. However, microbial absorbed ^{15}N should have been turnover to the soil during the experimental period, making them available for plants to uptake.

Carbon isotope discrimination was lower under both drought conditions in alfalfa and only under moderate drought conditions in red clover compared to well-watered conditions in our study. CID has been a proposed method and technique for evaluating water use efficiency (WUE) in C_3 plants like alfalfa (Raeini-Sarjaz et al. 1998). Plants under drought stress have been thought to utilize water more efficiently as a drought tolerance mechanism (Kaler et al. 2018). The CID is inversely proportionate with WUE (Hubick et al. 1986; Moghaddam et al. 2013), suggesting alfalfa under moderate and severe drought conditions and red clover under moderate drought stress had higher WUE compared to well-watered plants.

Soil extracellular enzyme activity (EEA) strongly predicts soil health, providing the leading force for nutrient cycling, including C, N, and P (Burns et al. 2013). To date, the effects of drought on soil EEA have been controversial. A meta-analysis by (Ren et al. 2017) reports a steep decrease in oxidoreductase activity while a general increase in hydrolase activities under limited precipitation. On the other hand, Geisseler et al. (2011) concluded that soil EEAs significantly increased under drought conditions. Our study found that drought generally did not affect soil EEA except for a significant decrease in activity of NAG under both 40% and 20% FC treatment in alfalfa and a significant reduction of C-acquisition hydrolase (Cello) under 20% FC

in red clover pots. The reduction of these specific enzymes is probably correlated to the availability of soil organic compounds (SOC), which is a common limiting factor for microbial growth in soils. This variation in enzymatic activity is highly associated with plant traits. The PCA analysis shows that both NAG and Cello are positively correlated with root biomass and architecture. Kanté et al. (2021) noted that enzyme activity is directly proportional to root biomass and length, thus associated with root growth as it will increase rhizodeposition through sloughing-off root border cells (Fustec et al. 2010). Furthermore, these plant-specific changes suggest that drought and plant composition may affect specific enzyme classes. Studies on the effect of plant species and variety on enzyme activity under drought periods are suggested to cause changes in soil EEAs (Beier et al. 2012; Steinauer et al. 2015). Drought influences root growth and root exudation, resulting in changes in the microbial community structure in the soil and rhizosphere. The change in microbial community structure prompts changes in soil enzyme activities (Trasar-Cepeda et al. 2008; Kabiri et al. 2016).

Drought affects root traits and soil nutrient availability (de Vries et al. 2020; Thilakarathna et al. 2016). We found an increased soil available nitrogen following severe drought stress in alfalfa and red clover. Among the N-containing exudates, ammonium and different amino acids are the major forms of N deposited by legumes contributing the majority of N retrieved in soil (Paynel et al. 2008; Lesuffleur et al. 2007). However, we observed that nitrate is more abundant in the soil than ammonium, likely due to the subsequent nitrification. Nitrate is not an N form actively released by legumes and thus does not naturally contribute to the soil-available N through active rhizodeposition. Drought induces root and nodule senescence, potentially releasing some organic N compounds. These organic N compounds convert into ammonium and further into nitrate, increasing available N in the soil following drought (Wichern et al. 2008; Fustec et al. 2010). Finally, the fate of the released N by legumes can also undergo different pathways; re-uptake by plants, loss through denitrification, and immobilization by soil microorganisms (Näsholm et al. 2009; Cameron et al. 2013).

Although past work has shown that drought negatively impacts plant physiological parameters and influences the shift in microbial community composition (Naylor and Coleman-Derr 2018; Bogati and Walczak 2022), many of these investigations have been conducted separately. In this study, we synergistically investigated how bacterial communities are structured in both the bulk and rhizosphere habitats as affected by plant physiological changes in two forage

legume species under drought conditions. Plants undergo several physiological changes to conserve water and adapt to drought stress. Here we show the effects of drought stress on plant physiological parameters, SNF, plant growth, and N rhizodeposition, which may then affect the plants' associated bacterial communities and their recruitment in the root zone.

The distinct separation of rhizosphere bacterial communities from the bulk soil in both forage legume species suggests that plant root exudates recruit a subset of bacteria from the bulk soil into the root zone (Finkel et al. 2017), and this recruitment is affected by a wide range of host and environmental conditions (Gaiero et al. 2013; Fitzpatrick et al. 2018). Here we observed plant-host differential responses of drought effects on bacterial diversity and community composition. Although not statistically significant, we observed a trend in reduced bacterial diversity in alfalfa and red clover bulk and alfalfa rhizosphere soils, but this trend was not observed in the red clover rhizosphere. The negative effect of drought on bacterial diversity was previously reported (Preece et al. 2019). However, some previous studies indicate no effects of drought on bacterial diversity (Acosta-Martínez et al. 2014; Bachar et al. 2010; Tóth et al. 2017). There are several potential reasons why bacterial diversity was not significantly impacted. In our study, three-week drought exposure might not have been long enough to cause significant changes in bacterial diversity. Other potential reasons could be that bacteria live in complex interacting communities with other microbiota. Some bacteria might produce exopolysaccharides (Khan and Bano 2019) that retain soil moisture, facilitating microsite formation, benefitting neighbouring bacteria and thus preserving bacterial diversity.

Previous studies have shown the sensitivity of Acidobacteria to drought with a decrease in abundance in the soil (Acosta-Martínez et al. 2014; Barnard et al. 2013; Maestre et al. 2015). Some studies have also demonstrated the enrichment of Acidobacteria under drought conditions (Yuste et al. 2014; Naylor and Coleman-Derr 2018). Acidobacteria were previously shown to be physiologically diverse (Huber et al. 2022); species under this group responded differently in varying conditions, which may be the possible reason for their differential responses in drought events. Genomic analysis unravelled the role of Acidobacteria in soil nitrogen cycling (Ward et al. 2009). Thus, in our study, the reduction of the relative abundances of soil Acidobacteria under drought in alfalfa was likely related to the reduction of N-acetylglucosaminadase involved in nitrogen cycling in the soil.

Enrichment of *Nocardioidaceae*, under phylum Actinobacteria, in the red clover rhizosphere is consistent with the results of several studies on the enrichment of Actinobacteria under drought stress (Bouskill et al. 2016; Kavamura et al. 2013; Santos-Medellín et al. 2021; Xu et al. 2018). Several factors are thought to contribute to the enrichment of this group; Actinobacteria are gram-positive, and many are monoderms, where the thick peptidoglycan cell wall and accumulation of osmolytes make this group more tolerant to desiccation (Hartman and Tringe 2019). Many members of Actinobacteria are known to produce siderophores and other secondary metabolites; recruitment may prove beneficial to plants for survival in stressful conditions (Lewin et al. 2016). While red clover demonstrated enriched taxa in the rhizosphere, this trend was not observed in the alfalfa rhizosphere, possibly reflecting differences in exudation patterns under drought stress.

While the relative abundances of N_2 fixing bacterial composition were not significantly impacted by drought exposure in the bulk and rhizosphere soil, we observed a significant increase in the relative abundance of Proteobacteria (order Rhizobiales being the most abundant taxon) in the bulk soil of alfalfa. The members of Proteobacteria, particularly Rhizobiales, may have been out-competed by other taxa that have a competitive advantage in the rhizosphere under drought conditions. Alternatively, drought may have altered the root exudates to favor the recruitment of other taxa, which led to the migration of Rhizobiales to the bulk soil that was more favorable for their growth.

We did not observe significant shifts in bacterial community structure subjected to drought. The effect of drought on microbial communities is complex and is associated with a range of factors, including frequency, intensity, and duration of drought (Naylor and Coleman-Derr 2018; Schimel 2018). The plant/soil microbiome associated with the plant host species being investigated in our study is likely generally tolerant to the duration of drought exposure and can bounce back after the exposure showing a resilient community. We recommend future research on the plant-soil microbiome in plant developmental stages and multiple time points across various host plant species under longer drought exposure.

This comprehensive study found the positive and negative effects of moderate and severe drought stress on aboveground (e.g., plant physiological parameters, shoot growth, shoot nitrogen, water use efficiency, SNF) and belowground (e.g., nodulation, root phenotypes, soil enzyme activities, soil N availability, soil microbiome) parameters of two forage legumes. In conclusion,

severe drought had significant negative effects on nodulation, root and shoot growth, and SNF. Soil available N was significantly increased under severe drought conditions. The extracellular enzyme assay showed that drought stress reduced the N-acetyl-glucosaminidase in alfalfa and β-D cellobiosidase activity in red clover. Microbiome data showed differential responses of the two forage plant species under drought conditions. While drought did not affect β-diversity in either plant host species, α-diversity was affected in alfalfa. Furthermore, we observed a decrease in the relative abundances of Acidobacteria in alfalfa, whereas, enrichment of *Nocardiodes* in red clover. Overall results indicate that drought has deleterious effects on nodulation, plant growth, and carbon and nitrogen cycling enzyme, while positively impacting soil nitrogen availability and some specific soil microbial taxa. However, the impact of drought stress on nodulation, plant physiological parameters, SNF, soil nitrogen availability and microbial diversity was also dependent on the forage legume species. The current study will contribute to understanding the effect of drought-impaired SNF on soil nitrogen availability, microbial diversity, and relationships between the different traits measured.

References

Acosta-Martínez V, Cotton J, Gardner T, Moore-Kucera J, Zak J, Wester D, Cox S (2014) Predominant bacterial and fungal assemblages in agricultural soils during a record drought/heat wave and linkages to enzyme activities of biogeochemical cycling. Appl Soil Ecol 84:69–82. https://doi.org/10.1016/j.apsoil.2014.06.005

Ashraf M, O'Leary JW (1996) Effect of drought stress on growth, water relations, and gas exchange of two lines of sunflower differing in degree of salt tolerance. Int J Plant Sci 157:729–732. https://doi.org/10.1086/297395

Bachar A, Al-Ashhab A, Soares MIM, Sklarz MY, Angel R, Ungar ED, Gillor O (2010) Soil microbial abundance and diversity along a low precipitation gradient. Microb Ecol 60:453–461. https://doi.org/10.1007/S00248-010-9727-1

Barnard RL, Osborne CA, Firestone MK (2013) Responses of soil bacterial and fungal communities to extreme desiccation and rewetting. ISME J 7:2229–2241. https://doi.org/10.1038/ISMEJ.2013.104

Basak R, Wahid KA, Dinh A, Soolanayakanahally R, Fotouhi R, Mehr AS (2020) Rapid and efficient determination of relative water contents of crop leaves using electrical impedance spectroscopy in vegetative growth stage. Remote Sens 12:1753. https://doi.org/10.3390/rs12111753

Begum N, Qin C, Ahanger MA, Raza S, Khan MI, Ashraf M, Ahmed N, Zhang L (2019) Role of arbuscular mycorrhizal fungi in plant growth regulation: implications in abiotic stress tolerance. Front Plant Sci 10:1068. https://doi.org/10.3389/fpls.2019.01068

Beier C, Beierkuhnlein C, Wohlgemuth T, Penuelas J, Emmett B, Körner C, de Boeck H, Christensen JH, Leuzinger S, Janssens IA et al (2012) Precipitation manipulation experiments–challenges and recommendations for the future. Ecol Lett 15:899–911. https://doi.org/10.1111/j.1461-0248.2012.01793.x

Berg G, Grube M, Schloter M, Smalla K (2014) Unraveling the plant microbiome: looking back and future perspectives. Front Microbiol 5:148. https://doi.org/10.3389/fmicb.2014.00148

Berg G, Rybakova D, Grube M, Köberl M (2016) The plant microbiome explored: implications for experimental botany. J Exp Bot 67:995–1002. https://doi.org/10.1093/jxb/erv466

Bogati K, Walczak M (2022) The impact of drought stress on soil microbial community, enzyme activities and plants. Agron 12:1–26. https://doi.org/10.3390/agronomy12010189

Bouskill NJ, Wood TE, Baran R, Ye Z, Bowen BP, Lim HC, Zhou J, Van Nostrand JD, Nico P, Northen TR et al (2016) Belowground response to drought in a tropical forest soil. I. changes in microbial functional potential and metabolism. Front Microbiol 7:525. https://doi.org/10.3389/fmicb.2016.00525

Burns RG (1982) Enzyme activity in soil: location and a possible role in microbial ecology. Soil Biol Biochem 14:423–427. https://doi.org/10.1016/0038-0717(82)90099-2

Burns RG, DeForest JL, Marxsen J, Sinsabaugh RL, Stromberger ME, Wallenstein MD, Weintraub MN, Zoppini A (2013) Soil enzymes in a changing environment: current knowledge and future directions. Soil Biol Biochem 58:216–234. https://doi.org/10.1016/j.soilbio.2012.11.009

Cameron KC, Di HJ, Moir JL (2013) Nitrogen losses from the soil/plant system: a review. Ann Appl Biol 162:145–173. https://doi.org/10.1111/aab.12014

Cavaglieri L, Orlando J, Etcheverry M (2009) Rhizosphere microbial community structure at different maize plant growth stages and root locations. Microbiol Res 164:391–399. https://doi.org/10.1016/j.micres.2007.03.006

Chaparro JM, Badri DV, Bakker MG, Sugiyama A, Manter DK, Vivanco JM (2013) Root exudation of phytochemicals in Arabidopsis follows specific patterns that are developmentally programmed and correlate with Soil Microbial functions. PLoS ONE 8:e55731. https://doi.org/10.1371/journal.pone.0055731

Chaparro JM, Badri DV, Vivanco JM (2014) Rhizosphere microbiome assemblage is affected by plant development. ISME J 8:790–803. https://doi.org/10.1038/ismej.2013.196

Chong J, Liu P, Zhou G, Xia J (2020) Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. Nat Protoc 15:799–821. https://doi.org/10.1038/s41596-019-0264-1

Christie BR, Bennett RJ (1984) OAC Minto Alfafla. Can J Plant Sci 64:419–421

Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, Brown CT, Porras-Alfaro A, Kuske CR, Tiedje JM (2014) Ribosomal database project: data and tools for high throughput rRNA analysis. Nucleic Acid Res 42:D633–D642. https://doi.org/10.1093/nar/gkt1244

de Freitas IC, Ferreira EA, Alves MA, de Oliveira JC, Frazão LA (2023) Growth, nodulation, production, and physiology of leguminous plants in integrated production systems. Agrosyst Geosci Environ 6:e20343. https://doi.org/10.1002/agg2.20343

De Haan RL, Schuiteman MA, Vos RJ (2017) Residual soil nitrate content and profitability of five cropping systems in northwest Iowa. PLoS ONE 12:e0171994. https://doi.org/10.1371/journal.pone.0171994

De Vries FT, Wallenstein MD (2017) Below-ground connections underlying above-ground food production: a framework for optimising ecological connections in the rhizosphere. J Ecol 105:913–920. https://doi.org/10.1111/1365-2745.12783

De Vries FT, Griffiths RI, Knight CG, Nicolitch O, Williams A (2020) Harnessing rhizosphere microbiomes for drought-resilient crop production. Science 368:270–274

del Castillo LD, Hunt S, Layzell DB (1994) The role of oxygen in the regulation of nitrogenase activity in drought-stressed soybean nodules. Plant Physiol 106:949–955

Dennis PG, Miller AJ, Hirsch PR (2010) Are root exudates more important than other sources of rhizodeposits in structuring rhizosphere bacterial communities? FEMS Microbiol Ecol 72:313–327. https://doi.org/10.1111/j.1574-6941.2010.00860.x

Dey R, Lewis SC, Arblaster JM, Abram NJ (2019) A review of past and projected changes in Australia's rainfall. Wiley Interdiscip Rev Clim Change 10:e00577. https://doi.org/10.1002/wcc.577

Enebe MC, Babalola OO (2018) The influence of plant growth-promoting rhizobacteria in plant tolerance to abiotic stress: a survival strategy. Appl Microbiol Biotech 102:7821–7835. https://doi.org/10.1007/s00253-018-9214-z

Fageria NK (2014) Nitrogen harvest index and its association with crop yields. J Plant Nutr 37:795–810. https://doi.org/10.1080/01904167.2014.881855

Farooq M, Gogoi N, Barthakur S, Baroowa B, Bharadwaj N, Alghamdi SS, Siddique KHM (2017) Drought stress in grain legumes during reproduction and grain filling. J Agro Crop Sci 203:81–102. https://doi.org/10.1111/jac.12169

Farooq M, Wahid A, Kobayashi N, Fujita D, Basra SMA (2009) Plant drought stress: effects, mechanisms and management. Agron Sustain Dev 29:185–212. https://doi.org/10.1051/agro:2008021

Fernández-Luqueño F, Dendooven L, Munive A, Corlay-Chee L, Serrano-Covarrubias LM, Espinosa-Victoria D (2008) Micro-morphology of common bean (*Phaseolus vulgaris* L.) nodules undergoing senescence. Acta Physiol Plant 30:545–552. https://doi.org/10.1007/s11738-008-0153-7

Fierer N, Schimel JP, Holden PA (2003) Variations in microbial community composition through two soil depth profiles. Soil Biol Biochem 35:167–176. https://doi.org/10.1016/s0038-0717(02)00251-1

Finkel OM, Castrillo G, Paredes SH, González IS, Dangl JL (2017) Understanding and exploiting plant beneficial microbes. Curr Opin Plant Biol 38:155–163. https://doi.org/10.1016/j.pbi.2017.04.018

Fitzpatrick CR, Copeland J, Wang PW, Guttman DS, Kotanen PM, Johnson MTJ (2018) Assembly and ecological function of the root microbiome across angiosperm plant species. PNAS 115:E1157–E1165. https://doi.org/10.1073/pnas.1717617115

Fustec J, Lesuffleur F, Mahieu S, Cliquet JB (2010) Nitrogen rhizodeposition of legumes. A review. Agron Sustain Dev 30:57–66

Gaiero JR, McCall CA, Thompson KA, Day NJ, Best AS, Dunfield KE (2013) Inside the root microbiome: bacterial root endophytes and plant growth promotion. Am J Bot 100:1738–1750. https://doi.org/10.3732/ajb.1200572

Geisseler D, Horwath WR, Scow KM (2011) Soil moisture and plant residue addition interact in their effect on extracellular enzyme activity. Pedobiologia (Jena) 54:71–78. https://doi.org/10.1016/j.pedobi.2010.10.001

German DP, Weintraub MN, Grandy AS, Lauber CL, Rinkes ZL, Allison SD (2011) Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. Soil Biol Biochem 43:1387–1397. https://doi.org/10.1016/j.soilbio.2011.03.017

González EM, Larrainzar E, Marino D, Wienkoop S, Gil-Quintana E, Arrese-Igor C (2015) Physiological responses of N2-fixing legumes to water limitation. In: Sulieman S, Tran LS (eds) Legume nitrogen fixation in a changing environment. Springer International Publishing, pp 5–33. https://doi.org/10.1007/978-3-319-06212-9_2

Hartman K, Tringe SG (2019) Interactions between plants and soil shaping the root microbiome under abiotic stress. Biochem J 476:2705–2724. https://doi.org/10.1042/BCJ20180615

Herrmann L, Chotte JL, Thuita M, Lesueur D (2014) Effects of cropping systems, maize residues application and N fertilization on promiscuous soybean yields and diversity of native rhizobia in Central Kenya. Pedobiologia (Jena) 57:75–85. https://doi.org/10.1016/j.pedobi.2013.12.004

Hewins DB, Fatemi F, Adams B, Carlyle C, Chang SX, Bork EW (2015) Grazing, regional climate and soil biophysical impacts on microbial enzyme activity in grassland soil of Western Canada. Pedobiologia (Jena) 58:201–209. https://doi.org/10.1016/j.pedobi.2015.10.003

Hopkins A, Del Prado A (2007) Implications of climate change for grassland in Europe: impacts, adaptations and mitigation options: a review. Grass Forage Sci 62:118–126. https://doi.org/10.1111/j.1365-2494.2007.00575.x

Huber KJ, Vieira S, Sikorski J, Wüst PK, Fösel BU, Gröngröft A, Overmann J (2022) Differential response of acidobacteria to water content, soil type, and land use during an extended drought in African savannah soils. Front Microbiol 13:750456. https://doi.org/10.3389/FMICB.2022.750456/FULL

Hubick KT, Farquhar GD, Shorter R (1986) Correlation between water-use efficiency and carbon isotope discrimination in diverse peanut (Arachis) germplasm. Aust J Plant Physiol 13:803–816

Jacques C, Girodet S, Leroy F, Pluchon S, Salon C, Prudent M (2022) Memory acclimation of water stress in pea rely on root system's plasticity and plant's ionome modulation. Front Plant Sci 13:1089720. https://doi.org/10.3389/fpls.2022.1089720

Jaleel CA, Manivannan P, Wahid A, Farooq M, Somasundaram R, Panneerselvam R (2009) Drought stress in plants: a review on morphological characteristics and pigments composition. Int J Agric Biol 11:100–105

Johnson DW, Dijkstra FA, Cheng W (2007) The effects of *Glycine max* and *Helianthus annuus* on nutrient availability in two soils. Soil Biol Biochem 39:2160–2163. https://doi.org/10.1016/j.soilbio.2007.01.036

Jones DL, Nguyen C, Finlay RD (2009) Carbon flow in the rhizosphere: carbon trading at the soil-root interface. Plant Soil 321:5–33. https://doi.org/10.1007/s11104-009-9925-0

Kabiri V, Raiesi F, Ghazavi MA (2016) Tillage effects on soil microbial biomass, SOM mineralization and enzyme activity in a semi-arid calcixerepts. Agric Ecosyst Environ 232:73–84. https://doi.org/10.1016/j.agee.2016.07.022

Kaler AS, Bazzer SK, Sanz-Saez A, Ray JD, Fritschi FB, Purcell LC (2018) Carbon isotope ratio fractionation among plant tissues of soybean. Plant Phenome J 1:1–6. https://doi.org/10.2135/tppj2018.04.0002

Kang Y, Han Y, Torres-Jerez I, Wang M, Tang Y, Monteros M, Udvardi M (2011) System responses to long-term drought and re-watering of two contrasting alfalfa varieties. Plant J 68:871–889. https://doi.org/10.1111/j.1365-313X.2011.04738.x

Kanté M, Riah-Anglet W, Cliquet JB, Trinsoutrot-Gattin I (2021) Soil enzyme activity and stoichiometry: linking soil microorganism resource requirement and legume carbon rhizodeposition. Agron 11:2131. https://doi.org/10.3390/agronomy11112131

Kaspar TC, Taylor HM, Shibles RM (1984) Taproot-elongation rates of soybean cultivars in the glasshouse and their relation to field rooting depth. Crop Sci 24:916–920. https://doi.org/10.2135/cropsci1984.0011183x002400050021x

Kasper S, Christoffersen B, Soti P, Racelis A (2019) Abiotic and biotic limitations to nodulation by leguminous cover crops in south Texas. Agric 9:209. https://doi.org/10.3390/agriculture9100209

Kaushal M, Wani SP (2016) Plant-growth-promoting rhizobacteria: drought stress alleviators to ameliorate crop production in drylands. Ann Microbiol 66:35–42. https://doi.org/10.1007/s13213-015-1112-3

Kavamura NV, Taketani RG, Lançoni MD, Andreote FD, Mendes R, de Melo SI (2013) Water regime influences bulk soil and rhizosphere of *Cereus jamacaru* bacterial communities in the Brazilian Caatinga biome. PLoS ONE 8:e73606. https://doi.org/10.1371/journal.pone.0073606

Kers JG, Saccenti E (2021) The power of microbiome studies: some considerations on which alpha and beta metrics to use and how to report results. Front Micro 12: 796025

Khan N, Bano A (2019) Exopolysaccharide producing rhizobacteria and their impact on growth and drought tolerance of wheat grown under rainfed conditions. PLoS ONE 14:e0222302. https://doi.org/10.1371/journal.pone.0222302

Kuhlgert S, Austic G, Zegarac R, Osei-Bonsu I, Hoh D, Chilvers MI, Roth MG, Bi K, TerAvest D, Weebadde P, Kuhlgert S, Austic G, Zegarac R, Osei-Bonsu I, Hoh D, Chilvers MI, Roth MG, Bi K, TerAvest D, Weebadde P, Kramer DM (2016) MultispeQ beta: a tool for large-scale plant phenotyping connected to the open photosynQ network. R Soc Open Sci 3:160592. https://doi.org/10.1098/rsos.160592

Kunert KJ, Vorster BJ, Fenta BA, Kibido T, Dionisio G, Foyer CH (2016) Drought stress responses in soybean roots and nodules. Front Plant Sci 7:1015. https://doi.org/10.3389/fpls.2016.01015

Laranjo M, Alexandre A, Oliveira S (2014) Legume growth-promoting rhizobia: an overview on the *Mesorhizobium* Genus. Microbiol Res 169:2–17. https://doi.org/10.1016/j.micres.2013.09.012

Lau JA, Lennon JT, Kellogg WK, Karl DM (2012) Rapid responses of soil microorganisms improve plant fitness in novel environments. PNAS 109:14058–14062. https://doi.org/10.1073/pnas.1202319109

Lesk C, Rowhani P, Ramankutty N (2016) Influence of extreme weather disasters on global crop production. Nature 529:84–87. https://doi.org/10.1038/NATURE16467

Lesuffleur F, Paynel F, Bataillé MP, Le Deunff E, Cliquet JB (2007) Root amino acid exudation: measurement of high efflux rates of glycine and serine from six different plant species. Plant Soil 294:235–246. https://doi.org/10.1007/s11104-007-9249-x

Lewin GR, Carlos C, Chevrette MG, Horn HA, McDonald BR, Stankey RJ, Fox BG, Currie CR (2016) Evolution and ecology of Actinobacteria and their bioenergy applications. Annu Rev Microbiol 70:235–254. https://doi.org/10.1146/annurev-micro-102215-095748

Liu F, Jensen CR, Andersen MN (2005) A review of drought adaptation in crop plants: changes in vegetative and reproductive physiology induced by ABA-based chemical signals. Aust J Agric Res 56:1245–1252. https://doi.org/10.1071/AR05062

Liyanage DK, Chathuranga I, Mori BA, Thilakarathna MS (2022) A simple, semi- automated, gravimetric method to simulate drought stress on plants. Agronomy 12:349. https://doi.org/10.3390/agronomy12020349

Liyanage DK, Torkamaneh D, Belzile F, Balasubramanian P, Hill B, Thilakarathna MS (2023) The genotypic variability among short-season soybean cultivars for nitrogen fixation under drought stress. Plants 12:1004. https://doi.org/10.3390/plants12051004

Lumactud RA, Dollete D, Liyanage DK, Szczyglowski K, Hill B, Thilakarathna MS (2023) The effect of drought stress on nodulation, plant growth, and nitrogen fixation in soybean during early plant growth. J Agron Crop Sci 209:345–354. https://doi.org/10.1111/jac.12627

Maestre FT, Delgado-Baquerizo M, Jeffries TC, Eldridge DJ, Ochoa V, Gozalo B, Quero JL, García-Gómez M, Gallardo A, Ulrich W, Maestre FT, Delgado-Baquerizo M, Jeffries TC, Eldridge DJ, Ochoa V, Gozalo B, Quero JL, García-Gómez M, Gallardo A, Ulrich W, Bowker MA, Arredondo T, Barraza-Zepeda C, Bran D, Florentino A, Gaitán J, Gutiérrez JR, Huber-Sannwald E, Jankju M, Mau RL, Miriti M, Naseri K, Ospina A, Stavi I, Wang D, Woods NN, Yuan X, Zaady E, Singh BK (2015) Increasing aridity reduces soil microbial diversity and abundance in global drylands. PNAS 112:15684–15689. https://doi.org/10.1073/pnas.1516684112

Mak M, Babla M, Xu SC, O'Carrigan A, Liu XH, Gong YM, Holford P, Chen ZH (2014) Leaf mesophyll K^+, H^+ and Ca^{2+} fluxes are involved in drought-induced decrease in photosynthesis and stomatal closure in soybean. Environ Exp Bot 98:1–12. https://doi.org/10.1016/j.envexpbot.2013.10.003

Manavalan LP, Guttikonda SK, Phan Tran LS, Nguyen HT (2009) Physiological and molecular approaches to improve drought resistance in soybean. Plant Cell Physiol 50:1260–1276. https://doi.org/10.1093/pcp/pcp082

Marchin RM, Ossola A, Leishman MR, Ellsworth DS (2020) A simple method for simulating drought effects on plants. Front Plant Sci 10:1715. https://doi.org/10.3389/fpls.2019.01715

Marquez-Garcia B, Shaw D, Cooper JW, Karpinska B, Quain MD, Makgopa EM, Kunert K, Foyer CH (2015) Redox markers for drought-induced nodule senescence, a process occurring after drought-induced senescence of the lowest leaves in soybean (*Glycine max*). Ann Bot 116:497–510. https://doi.org/10.1093/aob/mcv030

McPherson MR, Wang P, Marsh EL, Mitchell RB, Schachtman DP (2018) Isolation and analysis of microbial communities in soil, rhizosphere, and roots in perennial grass experiments. J Vis Exp 137:e57932. https://doi.org/10.3791/57932

Micheletto S, Rodriguez-Uribe L, Hernandez R, Richins RD, Curry J, O'Connell MA (2007) Comparative transcript profiling in roots of *Phaseolus acutifolius* and *P. Vulgaris* under water deficit stress. Plant Sci 173:510–520. https://doi.org/10.1016/j.plantsci.2007.08.003

Miransari M, Riahi H, Eftekhar F, Minaie A, Smith DL (2013) Improving soybean (*Glycine max* L.) N_2 fixation under stress. J Plant Growth Regul 32:909–921. https://doi.org/10.1007/s00344013-9335-7

Moghaddam A, Raza A, Vollmann J, Ardakani MR, Wanek W, Gollner G, Friedel JK (2013) Carbon isotope discrimination and water use efficiency relationships of alfalfa genotypes under irrigated and rain-fed organic farming. Eur J Agron 50:82–89. https://doi.org/10.1016/j.eja.2013.05.010

Mouradi M, Bouizgaren A, Farissi M, Makoudi B, Kabbadj A, Very AA, Sentenac H, Qaddoury A, Ghoulam C (2016) Osmopriming improves seeds germination, growth, antioxidant responses and membrane stability during early stage of Moroccan alfalfa populations under water deficit. Chil J Agric Res 76:265–272. https://doi.org/10.4067/S0718-58392016000300002

Mwale SE, Ochwo-Ssemakula M, Sadik K, Achola E, Okul V, Gibson P, Edema R, Singini W, Rubaihayo P (2017) Response of cowpea genotypes to drought stress in Uganda. Am J Plant Sci 8:720–733. https://doi.org/10.4236/ajps.2017.84050

Näsholm T, Kielland K, Ganeteg U (2009) Uptake of organic nitrogen by plants. New Phytol 182:31–48. https://doi.org/10.1111/j.1469-8137.2008.02751.x

Naya L, Ladrera R, Ramos J, González EM, Arrese-Igor C, Minchin FR, Becana M (2007) The response of carbon metabolism and antioxidant defenses of alfalfa nodules to drought stress and to the subsequent recovery of plants. Plant Physiol 144:1104–1114. https://doi.org/10.1104/pp.107.099648

Naylor D, Coleman-Derr D (2018) Drought stress and root-associated bacterial communities. Front Plant Sci 8:2223. https://doi.org/10.3389/fpls.2017.02223

Nishida H, Suzaki T (2018) Nitrate-mediated control of root nodule symbiosis. Curr Opin Plant Biol 44:129–136. https://doi.org/10.1016/j.pbi.2018.04.006

Niu X, Song L, Xiao Y, Ge W (2018) Drought-tolerant plant growth-promoting rhizobacteria associated with foxtail millet in a semi-arid and their potential in alleviating drought stress. Front Microbiol 8:2580. https://doi.org/10.3389/fmicb.2017.02580

Parida AK, Dagaonkar VS, Phalak MS, Umalkar GV, Aurangabadkar LP (2007) Alterations in photosynthetic pigments, protein and osmotic components in cotton genotypes subjected to short-term drought stress followed by recovery. Plant Biotechnol Rep 1:37–48. https://doi.org/10.1007/s11816-006-0004-1

Paynel F, Lesuffleur F, Bigot J, Diquélou S, Cliquet JB (2008) A study of ^{15}N transfer between legumes and grasses. Agron Sustain Dev 28:281–290. https://doi.org/10.1051/agro:2007061

Pohlert T (2022) PMCMRplus: calculate pairwise multiple comparisons of mean rank sums extended. R package version 1.9.6. https://CRAN.R-project.org/package=PMCMRplus. Accessed 20 Aug 2022

Poorter H, Fiorani F, Stitt M, Schurr U, Finck A, Gibon Y, Usadel B, Munns R, Atkin OK, Tardieu F, Pons TL (2012) The art of growing plants for experimental purposes: a practical guide for the plant biologist. Funct Plant Biol 39:821–838. https://doi.org/10.1071/FP12028

Preece C, Peñuelas J (2016) Rhizodeposition under drought and consequences for soil communities and ecosystem resilience. Plant Soil 409:1–7. https://doi.org/10.1007/s11104-016-3090-z

Preece C, Verbruggen E, Liu L, Weedon JT, Peñuelas J (2019) Effects of past and current drought on the composition and diversity of soil microbial communities. Soil Biol Biochem 131:28–39. https://doi.org/10.1016/j.soilbio.2018.12.022

R Core Team (2023) R: A language and environment for tatistical computing. R Foundation for Statistical Computing, Vienna. https://www.R-project.org/. Accessed 15 Mar 2023

Raeini-Sarjaz M, Barthakur NN, Arnold NP, Jones PJH (1998) Water stress, water use efficiency, carbon isotope discrimination and leaf gas exchange relationships of the bush bean. J Agron Crop Sci 180:173–179. https://doi.org/10.1111/j.1439-037x.1998.tb00387.x

Rehman A, Nautiyal CS (2002) Effect of drought on the growth and survival of the stress-tolerant bacterium *Rhizobium* sp. NBRI2505 *sesbania* and its drought-sensitive transposon Tn5 mutant. Curr Microbiol 45:368–377. https://doi.org/10.1007/s00284-002-3770-1

Ren C, Zhao F, Shi Z, Chen J, Han X, Yang G, Feng Y, Ren G (2017) Differential responses of soil microbial biomass and carbon-degrading enzyme activities to altered precipitation. Soil Biol Biochem 115:1–10. https://doi.org/10.1016/j.soilbio.2017.08.002

Rochon JJ, Doyle CJ, Greef JM, Hopkins A, Molle G, Sitzia M, Scholefield D, Smith CJ (2004) Grazing legumes in Europe: a review of their status, management, benefits, research needs and future prospects. Grass Forage Sci 59:197–214

Rognes T, Flouri T, Nichols B, Quince C, Mahé F (2016) VSEARCH: a versatile open source tool for metagenomics. PeerJ 4:e2584. https://doi.org/10.7717/peerj.2584/FIG-7

Rouached A, Slama I, Zorrig W, Jdey A, Cukier C, Rabhi M, Talbi O, Limami AM, Abdelly C (2013) Differential performance of two forage species, *Medicago truncatula* and *sulla carnosa*, under water-deficit stress and recovery. Crop Pasture Sci 64:254–264. https://doi.org/10.1071/CP13049

Rubiales D, Mikic A (2015) Introduction: legumes in sustainable agriculture. CRC Crit Rev Plant Sci 34:2–3. https://doi.org/10.1080/07352689.2014.897896

Saiya-Cork KR, Sinsabaugh RL, Zak DR (2002) The effects of long term nitrogen deposition on extracellular enzyme activity in an *Acer saccharum* forest soil. Soil Biol Biochem 34:1309–1315

Santos-Medellín C, Liechty Z, Edwards J, Nguyen B, Huang B, Weimer BC, Sundaresan V (2021) Prolonged drought imparts lasting compositional changes to the rice root microbiome. Nat Plants 7:1065–1077. https://doi.org/10.1038/s41477-021-00967-1

Schimel JP (2018) Life in Dry soils: effects of drought on soil microbial communities and processes. Annu Rev Ecol Evol Syst 49:409–432. https://doi.org/10.1146/annurev-ecolsys-110617-062614

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537–7541. https://doi.org/10.1128/AEM.01541-09

Schwalm CR, Anderegg WRL, Michalak AM, Fisher JB, Biondi F, Koch G, Litvak M, Ogle K, Shaw JD, Wolf A, Schwalm CR, Anderegg WRL, Michalak AM, Fisher JB, Biondi F, Koch G, Litvak M, Ogle K, Shaw JD, Wolf A, Huntzinger DN, Schaefer K, Cook R, Wei

Y, Fang Y, Hayes D, Huang M, Jain A, Tian H (2017) Global patterns of drought recovery. Nature 548:202–205. https://doi.org/10.1038/nature23021

Serraj R (2003) Effects of drought stress on legume symbiotic nitrogen fixation: physiological mechanisms. Indian J Exp Biol 41:1136–1141

Serraj R, Sinclair TR, Purcell LC (1999) Symbiotic N_2 fixation response to drought. J Exp Bot 50:143–155. https://doi.org/10.1093/jxb/50.331.143

Serraj R, Vadez V, Sinclair T, Sinclair TR (2001) Feedback regulation of symbiotic N_2 fixation under drought stress. Agron 21:6–7. https://doi.org/10.1051/agro:2001153ï

Steinauer K, Tilman D, Wragg PD, Cesarz S, Cowles JM, Pritsch K, Reich PB, Weisser WW, Eisenhauer N (2015) Plant diversity effects on soil microbial functions and enzymes are stronger than warming in a grassland experiment. Ecol 96:99–112. https://doi.org/10.1890/14-0088.1

Thilakarathna MS, Cope KR (2021) Split-root assays for studying legume-rhizobia symbioses, rhizodeposition, and belowground nitrogen transfer in legumes. J Exp Bot 72:5285–5299. https://doi.org/10.1093/jxb/erab198

Thilakarathna MS, McElroy MS, Chapagain T, Papadopoulos YA, Raizada MN (2016) Belowground nitrogen transfer from legumes to non-legumes under managed herbaceous cropping systems. A review. Agron Sustain Dev 36:58. https://doi.org/10.1007/s13593-016-0396-4

Thilakarathna MS, Moroz N, Raizada MN (2017) A biosensor-based leaf punch assay for glutamine correlates to symbiotic nitrogen fixation measurements in legumes to permit rapid screening of rhizobia inoculants under control conditions. Front Plant Sci 08:1714. https://doi.org/10.3389/fpls.2017.01714

Thilakarathna MS, Raizada MN (2017) A meta-analysis of the effectiveness of diverse rhizobia inoculants on soybean traits under field conditions. Soil Biol Biochem 105:177–196. https://doi.org/10.1016/j.soilbio.2016.11.022

Thilakarathna MS, Raizada MN (2018) Visualizing glutamine accumulation in root systems involved in the legume–rhizobia symbiosis by placement on agar embedded with companion biosensor cells. Phytobiomes J 2:117–128. https://doi.org/10.1094/PBIOMES-07-18-0031-TA

Thilakarathna MS, Raizada MN (2019) A biosensor-based assay (*GlnLux*-Agar) shows defoliation triggers rapid release of glutamine from nodules and young roots of forage leguumes. Phytobiomes J 3:85–91. https://doi.org/10.1094/PBIOMES-03-19-0014-Rs

Tóth Z, Táncsics A, Kriszt B, Kröel-Dulay G, Ónodi G, Hornung E (2017) Extreme effects of drought on composition of the soil bacterial community and decomposition of plant tissue. Eur J Soil Sci 68:504–513. https://doi.org/10.1111/ejss.12429

Trasar-Cepeda C, Leirós MC, Gil-Sotres F (2008) Hydrolytic enzyme activities in agricultural and forest soils. Some implications for their use as indicators of soil quality. Soil Biol Biochem 40:2146–2155. https://doi.org/10.1016/j.soilbio.2008.03.015

Udvardi M, Poole PS (2013) Transport and metabolism in lgume rhizobia symbioses. Annu Rev Plant Biol 64:781–805. https://doi.org/10.1146/annurev-arplant

Valentine AJ, Benedito VA, Kang Y (2011) Legume nitrogen fixation and soil abiotic stress: from physiology to genomics and beyond. In: Foyer CH, Zhang H (eds) Nitrogen metabolism in plants in the post-genomic era, vol 42, Wiley-Blackwell Publishing, pp 207–248. https://doi.org/10.1002/9781444328608.ch9

Vaseva I, Akiscan Y, Demirevska K, Anders I, Feller U (2011) Drought stress tolerance of red and white clover-comparative analysis of some chaperonins and dehydrins. Sci Hortic 130:653–659. https://doi.org/10.1016/j.scienta.2011.08.021

Wang W, Wang C, Pan D, Zhang Y, Luo B, Ji J (2018) Effects of drought stress on photosynthesis and chlorophyll fluorescence images of soybean (*Glycine max*) seedlings. Int J Agric Biol Eng 11:196–201. https://doi.org/10.25165/j.ijabe.20181102.3390

Ward NL, Challacombe JF, Janssen PH, Henrissat B, Coutinho PM, Wu M, Xie G, Haft DH, Sait M, Badger J, Ward NL, Challacombe JF, Janssen PH, Henrissat B, Coutinho PM, Wu M, Xie G, Haft DH, Sait M, Badger J, Barabote RD, Bradley B, Brettin TS, Brinkac LM, Bruce D, Creasy T, Daugherty SC, Davidsen TM, DeBoy RT, Detter JC, Dodson RJ, Durkin AS, Ganapathy A, Gwinn-Giglio M, Han CS, Khouri H, Kiss H, Kothari SP, Madupu R, Nelson KE, Nelson WC, Paulsen I, Penn K, Ren Q, Rosovitz MJ, Selengut JD, Shrivastava S, Sullivan SA, Tapia R, Thompson LS, Watkins KL, Yang Qi, Yu C, Zafar N, Zhou L, Kuske CR (2009) Three genomes from the phylum Acidobacteria provide insight into the lifestyles of these microorganisms in soils. Appl Environ Microbiol 75:2046–2056. https://doi.org/10.1128/AEM.02294-08

Weiss S, Xu ZZ, Peddada S, Amir A, Bittinger K, Gonzalez A, Lozupone C, Zaneveld JR, Vázquez-Baeza Y, Weiss S, Xu ZZ, Peddada S, Amir A, Bittinger K, Gonzalez A, Lozupone C, Zaneveld JR, Vázquez-Baeza Y, Birmingham A, Hyde ER, Knight R (2017) Normalization and microbial differential abundance strategies depend upon data characteristics. Microbiome 5:27. https://doi.org/10.1186/s40168-017-0237-y

Wichern F, Eberhardt E, Mayer J, Joergensen RG, Müller T (2008) Nitrogen rhizodeposition in agricultural crops: methods, estimates and future prospects. Soil Biol Biochem 40:30–48. https://doi.org/10.1016/j.soilbio.2007.08.010

Xu L, Naylor D, Dong Z, Simmons T, Pierroz G, Hixson KK, Kim YM, Zink EM, Engbrecht KM, Wang Y, Xu L, Naylor D, Dong Z, Simmons T, Pierroz G, Hixson KK, Kim Y-M, Zink EM, Engbrecht KM, Wang Yi, Gao C, DeGraaf S, Madera MA, Sievert JA, Hollingsworth J, Birdseye D, Scheller HV, Hutmacher R, Dahlberg J, Jansson C, Taylor JW, Lemaux PG, Coleman-Derr D (2018) Drought delays development of the sorghum root microbiome and enriches for monoderm bacteria. PNAS 115:E4284–E4293. https://doi.org/10.1073/PNAS.1717308115

Yuste JC, Fernandez-Gonzales AJ, Fernandez-Lopez M, Ogaya R, Penuelas J, Sardans J, Lloret F (2014) Strong functional stability of soil microbial communities under semiarid Mediterranean conditions and subjected to long-term shifts in baseline precipitation. Soil Biol Biochem 69:223–233. https://doi.org/10.1016/j.soilbio.2013.10.045

Zahran HH (1999) Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. Microbiol Mol Biol Rev 63:968–989. https://doi.org/10.1128/mmbr.63.4.968-989.1999

Zargar SM, Gupta N, Nazir M, Mahajan R, Malik FA, Sofi NR, Shikari AB, Salgotra RK (2017) Impact of drought on photosynthesis: molecular perspective. Plant Gene 11:154–159. https://doi.org/10.1016/j.plgene.2017.04.003

Zhang C, Shi S, Liu Z, Yang F, Yin G (2018) Drought tolerance in alfalfa (*Medicago sativa* L.) varieties is associated with enhanced antioxidative protection and declined lipid peroxidation. J Plant Physiol 232:226–240. https://doi.org/10.1016/j.jplph.2018.10.023

Chapter 3 - Effect of defoliation intensity on symbiotic nitrogen fixation, soil nitrogen availability and soil microbial dynamics in forage legumes

Abstract

Forage legumes are critical for producing protein-rich high-quality pastures and for improving soil fertility through their ability to fix atmospheric nitrogen from the mutualistic relation with soil rhizobia bacteria. Forage legumes in pastures frequently undergo defoliation stress through grazing and mowing, which changes source-sink relationship between above-ground and below-ground tissues, potentially influencing key factors affecting their ability to fix nitrogen. In this greenhouse study, we evaluated the effects of two defoliation intensities on nodulation, root phenotypic traits, plant biomass, symbiotic nitrogen fixation, soil available nitrogen, soil enzyme activities, and soil microbial community structure of alfalfa (*Medicago sativa* L.) and red clover (*Trifolium pretense* L.). Plant defoliation treatments included a mild defoliation which removed half the above ground biomass, a severe defoliation that removed all but 2 cm of stubble and a non-defoliated control. Mild defoliation had a positive influence on the final shoot biomass in both legumes, but both mild and severe defoliation had a negative effect on nodulation and non-symbiotic root phenotypic traits, including root biomass. The symbiotic nitrogen fixation capacity was reduced in red clover under severe defoliation stress, whereas it was unaffected in alfalfa. Soil available nitrogen content was greater following severe defoliation in red clover compared to the mild and non-defoliation, but no changes were observed in alfalfa following defoliation. Severe defoliation significantly increased soil enzyme activities of β-1, 4-glucosidase, β-D-cellobiosidase, and phosphatase enzymes in both legumes. Finally, microbiome analysis showed an enrichment of the subgroup Gp3 from Acidobacteria following severe defoliation stress. Overall results suggest that defoliation intensity had a deleterious effect on root traits, a positive influence on C and phosphate extracellular enzyme activities, but varied influence on the shoot growth, symbiotic nitrogen fixation, and soil available nitrogen based on the forage legume type.

Keywords: forage legumes, defoliation, symbiotic nitrogen fixation, soil enzymes, available soil nitrogen, soil microbiome

3.1 Introduction

Forage legumes are important plants in a variety of cropping systems due to their capability to fix atmospheric N_2 in symbiosis with rhizobia bacteria, which renders them to provide nitrogen (N)-rich feed for livestock and also enhance soil health (Rochon et al., 2004.; Stagnari et al., 2017). Forage legumes improve soil N availability through the rhizodeposition of fixed N, thus minimizing the need for external N inputs (Ayres et al., 2007; Pirhofer-Walzl et al. 2012; Høgh-Jensen and Schjoerring 2001). However, plant productivity and persistence can be adversely influenced by defoliation, which alters the source-sink relationships between the aboveground and belowground parts for carbon (C) and N, favoring one over the other depending on the remaining photosynthetic tissues, which can influence the overall plant nitrogen fixation (Baysdorfer and Bassham, 1985; Black et al., 2009; Proulx and Naeve, 2009).

Defoliation is the removal of aboveground plant tissues, mainly associated with grazing and pest damage (Thilakarathna et al. 2016), and alters the balance between aboveground and belowground biomass, influencing the efficiency of the remaining photosynthetic tissues in capturing sunlight for energy (Gordon et al., 1990; Richards, 1993). The reduction in photosynthesis due to defoliation restricts the photosynthates supply to root and nodules (King and Purcell, 2001) and the metabolic rate of nodules declines following defoliation which reduces N fixation (Aranjuelo et al., 2015). Furthermore, defoliation can set off a myriad of physico-biochemical changes in nodules that can lead to reduced N fixation, potentially affecting other plant phenotypic and physiological traits. This includes reduced nitrogenase activity (del Castillo et al., 1994; Nishida and Suzaki, 2018), decreased oxygen permeability (Serraj 2003; González et al. 2015), reduction in glutamine synthetase (Jacobi et al. 1994), and enhanced tissue senescence (Vance et al. 1979). Defoliation can also trigger the exudation of fixed nitrogen through the root system of forage legumes immediately following defoliation (Thilakarathna and Raizada, 2019). In the long-term, senescence of belowground tissues, including roots and nodules, alters N availability in the rhizosphere and bulk soil (Hamilton et al., 2008; Carrillo et al., 2011; Thilakarathna and Raizada, 2019).

Plant roots are the primary conduits and source of organic C and other nutrient deposition to soil, which become available for rhizosphere bacteria (Hu et al., 2018; Ankati and Podile, 2019). Plants exhibit different rhizodeposition patterns depending on species, growth stage, health, and stress factors (Cavaglieri et al., 2009; Chaparro et al., 2013, 2014). Defoliation-induced changes

in plant root growth, nodulation, and SNF, potentially influence rhizodeposition of C and N (Hamilton et al., 2008; Carrillo et al., 2011), which are the main growth-limiting nutrients for soil microorganisms (Kanté et al., 2021; Macdonald et al., 2006; Ma et al., 2018). The rhizosphere microbiomes play a critical role in agroecosystems and are crucial for plant health and nutrition (Ma et al., 2018; Berg et al., 2016; Ling et al., 2022). They are responsible for nutrient cycling and organic matter turnover (Bausenwein et al., 2008). Microbes drive soil biochemical processes by releasing enzymes that catalyze various reactions (Bowles et al., 2014; Rao et al., 2014; Adetunji et al., 2020) and serve as an indicator of soil health (Liu et al., 2020; Bai et al., 2021).

Extensive research has been conducted to evaluate the effect of defoliation on the plant growth and above-ground physiological parameters in legume crops (Haagenson et al., 2003; Zhang et al., 2018; Kopture et al., 2023). However, less attention has been given to the effects of defoliation stress on the below-ground plant traits, and soil microbial community and biochemical processes, like extracellular enzyme activity. We hypothesize that defoliation impairs symbiotic N fixation (SNF) in forage legumes, which ultimately affects soil N availability, enzyme activities, and the microbial community. To this end, this study aimed to assess the effect of mild and severe defoliation on root growth, nodulation, SNF, extracellular enzyme activities, soil N availability, and soil microbiome in alfalfa and red clover.

3.2 Materials and Methods

3.2.1 Plant material and experimental design

Seeds of alfalfa variety OAC-Minto and red clover variety Juliet Double Cut (Christie and Bennett, 1984) were surface sterilized with 70% ethanol for 2 min, 2% NaOCl solution for 3 min and washed with six changes of autoclaved distilled water (Thilakarathna et al., 2017). Seedlings were germinated on sterile moistened filter paper in the dark at 28 ºC for two days. Five seedlings were planted into 6.5 L pots (21.7 cm height, 22 cm diameter) lined with low-density polyethylene (LPDE) plastic and filled with 1:2 field soil, collected from a wheat stubble field at the University of Alberta South Campus farm, and sand (QUIKRETE® Premium Play Sand, QUIKRETE, Atlanta, GA, USA). The soil-sand mixture was mixed for 10 minutes using a soil mixer (STOW mortar mixer, model M6-63). The mixture had a total N content of 7.36 mg/kg. Alfalfa and red clover seedlings were inoculated with 1 ml liquid inoculum of *Sinorhizobium meliloti* 1021 and *Rhizobium leguminosarum biovar trifolii* Mj 43, respectively (Thilakarathna and Raizada, 2018),

which are compatible rhizobia strains with these two forage legumes. Rhizobia inoculants were prepared by adjusting the optical density (OD_{595}) to 0.1. Pots were thinned to three seedlings per pot after one week of growth and reinoculated with corresponding rhizobia strains to ensure nodulation. Five planted pots of each species were prepared with sterile soil-sand mix and were left uninoculated to serve as non-fixing plants in calculating the nitrogen fixation capacity. Pots were arranged as randomized complete block designs with five replicates (n = 5) per defoliation treatment.

All plants were supplied with 50 ml of quarter-strength N-free Hoagland's nutrient solution twice per week (pH = 6.8, adjusted using KOH) (HOP03-50LT, Caisson Labs, UT). Plants were grown in the greenhouse at 24 ± 3°C and 16/8 h light/dark photoperiod until flowering stage. At the flowering stage, plants were subjected to three clipping treatments: mild defoliation (50% of the above-ground biomass was removed) by measuring from the base of the plant near the soil through halfway its shoot length and clipping the upper half, severe defoliation (almost 100% of the above-ground biomass was removed, leaving only 2 cm stubble from the ground level), and non-defoliation control (no defoliation). Defoliation treatments were imposed by clipping plants manually using scissors. Following defoliation treatments, plants were allowed to regrow for four weeks for recovery.

3.2.2 Measurement of soil available nitrogen

Plant Root Simulator (PRS) probes (PRS^{TM}; Western Ag Innovations, Saskatoon, SK, Canada) were buried into the soil (3 cation and anion pairs per pot) right after the defoliation treatments. After one week of burial, the PRS probes were retrieved and rinsed with milli-Q water to remove any adhering soil particles. The probes were placed in Ziploc bags on ice and sent to the Western Ag Innovations laboratory (Saskatoon, SK) for available nitrogen analysis. The PRS probes were eluted with 17.5 mL of 0.5 M HCl solution for 1 hour for ≥95% ions retrieval required for elemental determination. Blank probes were analyzed to ascertain that no N contamination occurred. Soil nitrogen-supply rates were expressed as mg N/10 cm^2/burial period (10 cm^2 representing the surface area of exchangeable resin) (Johnson et al., 2007). PRS burial was implemented again after four weeks of recovery for another week prior to the final harvest.

3.2.3 Shoot and root traits

Plants were harvested after the four-week recovery period and separated into shoots and roots. The root system of each plant was carefully shaken to remove excess soil and was washed free of soil and stored at 4 ºC in Ziploc bags. Roots were scanned with an Epson Expression 1640 scanner (Epson Canada Ltd., Markham, ON, Canada), and root architecture traits were obtained using WinRHIZO software (Regent Instruments Inc., Quebec City, QC, Canada), including root length, volume, and surface area (Thilakarathna et al., 2016). Nodules were removed from the roots and separated into single and cluster nodules for counting. Plant materials were dried at 60 ºC for three days for dry weight measurements.

3.2.4 Bulk and rhizosphere soil sampling

Bulk and rhizosphere soil sampling was performed following the protocol outlined by McPherson et al. (2018). After the recovery period, the bulk soil samples were collected using a 2.5 cm diameter, 12 cm long metal soil corer and stored in Ziplock bags at -20 ºC. For rhizosphere sample collection, 5 to 6 samples of lateral roots with residual soil still covering the root surface, each with ~12 cm length, were randomly selected from the root system to obtain rhizosphere samples and are kept in 50 ml sterile plastic tubes with 35 ml phosphate buffer at 4 ºC. The tubes containing lateral root clippings and buffer solution were vortexed for 2 min to loosen the soil. The roots were carefully removed, and the suspensions were centrifuged at 3000 g for 5 min at room temperature. Then, the supernatants were decanted, and pellets were resuspended in 1.5 ml phosphate buffer and vortexed. Approximately 2 ml of suspensions were transferred into new 2 ml microfuge tubes and centrifuged at room temperature at 16,000 g for 2 min. The supernatants were decanted, and the remaining soil pellets were stored at -20 ºC for microbial DNA extraction.

3.2.5 Extracellular enzymatic activity (EEA) assay

The extracellular enzyme activity (EEA) of the soil samples was measured following the protocol by Saiya-Cork et al. (2002) with modifications from German et al. (2011). The activity of the following four enzymes with significant roles in the cycling of soil C, N, and P were assayed: (1) cellulolytic enzymes β-1, 4-glucosidase (BG) and β-D-cellobiosidase (Cello) responsible for the release of glucose from cellobiose for microbial C acquisition; (2) phosphatase (Phos), responsible for the release of inorganic P from esters; and (3) glucosaminidase enzyme *N*-acetyl-

β glucosaminidase (NAG), which facilitates the breakdown of organic matter such as chitin of fungal cell wall, releasing N compounds (Hewins et al. 2015).

Each bulk soil sample was separated into two parts. One part was weighed for initial weight and oven-dried at 75 ºC for two days, and then the dry weight was recorded. The other part was used as a representative sample for the enzyme activity assay. A 2 g soil sample was resuspended and buffered with 125 ml of 50 mM acetate buffer (pH = ±7.2; CH_3COONa; CAS:127-09-3; BP333-500; Fisher Scientific; Toronto, ON, Canada). The solutions were kept homogenized using a magnetic stirrer. Next, 200 µl aliquots were dispensed from the suspension into black Costar 96-well microplates. The enzyme assays were conducted using a ratio of 2 g of soil per the standard 50 µl of 200 µM of substrate solution (Saiya-Cork et al., 2002). Blank wells received 50 µl of acetate buffer and 200 µl of sample suspension, while negative control wells received 50 µl of substrate solution and 200 µl of acetate buffer. Lastly, quench standard wells received 50 µl of standard MUB (10 µM 4-Methylumbelliferone), while reference standard wells received 50 µl standard MUB plus 200 µl acetate buffer. The assay plates were incubated for 5 h at room temperature and were read using a Spectramax M3 plate reader (Molecular Devices, LLC. San Jose, CA, USA) at 365 nm excitation and 450 nm emission. Activities were calculated on a per gram of dry soil basis using a standard equation (German et al., 2011).

3.2.6 ^{15}N isotope analysis for nitrogen fixation measurements

In order to measure nitrogen fixation using the ^{15}N isotope dilution technique, all plants were labeled with 25 ml of 0.5 mM $K^{15}NO_3$ solution (10 atom% ^{15}N; 348481-25G; Sigma Aldrich, Oakville, ON, Canada) following two and three weeks of growth. After each harvest, dried shoot materials were ground into powder using a SPEX SamplePrep 8000M Mixer/Mill ball grinder for 10 minutes, followed by Beadruptor (Beadruptor 12 Homogenizer, Omni International Inc.). A 5 mg subsample was measured from individual samples using a microbalance and was encapsulated in tin capsules (8 mm × 5 mm, D1008, standard weight, Isomass Scientific Inc.) (Liyanage et al., 2023). The ^{15}N and total N% of samples were analyzed using an Isotope Ratio Mass Spectrometer (IRMS) fitted with a Flash 2000 Elemental Analyzer (Thermo Fisher Scientific, Voltaweg, Netherlands) and Conflo IV (Thermo Fisher Scientific, Bremen, Germany) interface between the IRMS. The percentage of nitrogen derived from the atmosphere (%Ndfa) of shoot tissues was calculated using the following formula:

$$\%Ndfa = \left(1 - \frac{\text{atom\% } {}^{15}N \; excess_{(fixing\ plant)}}{\text{atom\% } {}^{15}N \; excess_{(non-fixing\ plant)}}\right) \times 100$$

Rhizobia uninoculated alfalfa and red clover plants (non-nodulating control) from the same experiments were used as the non-fixing references.

3.2.7 Bacterial DNA extraction, sequencing, and bioinformatics analyses

Total DNA was extracted from the soil samples using the Qiagen Dneasy PowerSoil Pro kit (Qiagen, Germany). Genomic DNA concentration and purity were validated using NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). DNA samples were sent to the Laval Genomics Platform (University of Laval, Quebec, Canada) for Illumina Miseq paired-end sequencing of the bacterial 16S rRNA V3-V4 region using primers — 341F 3'-GCCTACGGGNGGCWGCAG-5' and 806R 3'-ACTACHVGGGTATCTAATCC-5'. Nextera XT index kit (Illumina Inc. USA) was used to generate the amplicon libraries, observing the metagenomic sequencing library preparation protocol. Mothur pipeline (v.1.42) filtered the paired-end raw reads and produced contigs (Schloss et al., 2009). Chimeric sequences were removed using VSEARCH (Rognes et al., 2016). The sequences and OTUs were prepared using the RDP classifier database (Cole et al. 2014). MicrobiomeAnalyst (Chong et al., 2020) was used for further bioinformatics analysis. The sequences were rarified to the minimum library size implemented in MicrobiomeAnalyst (Weiss et al. 2017) to address the variability in sequencing depth among the samples. Raw sequences obtained from this project were deposited into the NCBI Sequence Read Archive under the accession number PRJNA1031707.

3.2.8 Data Analysis

Statistical analyses were performed in R v.4.23 (R core team 2023). The effect of different defoliation intensities on nodulation, plant biomass, root architecture, SNF, soil available N, soil extracellular enzyme activities, and microbial diversity and abundance were analyzed with ANOVA at $p<0.05$ with base R "aov" function after the data passed assumptions of normality and equal variances following Shapiro-Wilk test "shapiro.test" and Levene's test "leveneTest" in the car package version 3.1-2, respectively. The differences among the defoliation treatments were

assessed through the Tukey test ("TukeyHSD" in base R). Principal component analysis (PCA) was performed to visualize the multivariate effects of defoliation intensity on mean values of significantly affected plant traits and soil parameters. The PCA figures were created using the autoplot function in ggplot2 v.3.3.6, formatted with ggfortify v.0.4.16. MicrobiomeAnalyst was used to analyze alpha- and beta-diversity (Chong et al. 2020). The p-values were corrected for multiple factor testing using the Benjamini-Hochberg's False-Discovery Rate (FDR) in MicrobiomeAnalyst. Permutational multivariate analysis of variance (PERMANOVA) with Bray-Curtis dissimilarity was performed to identify differences in bacterial communities after defoliation stress.

3.3 Results

3.3.1 Effect of defoliation on nodulation

Mild and severe defoliation treatments significantly reduced the number of single nodules ($P<0.05$; Figs. 3.1A) and number of cluster nodules ($P<0.05$; Figs. 3.1C) in alfalfa, resulting in the overall reduction in total nodule number ($P<0.05$; Figs. 3.1E) compared to the non-defoliation control. In red clover, the number of single nodules and total number of nodules were significantly reduced under mild and severe defoliation ($P<0.05$; Figs. 3.1B, F), whereas the number of cluster nodules was reduced only under severe defoliation compared to the non-defoliated control ($P<0.05$; Figs. 3.1D). Defoliation also significantly reduced the dry weight of alfalfa nodules under both defoliation treatments ($P<0.05$; Figs. 3.1G), while only severe defoliation significantly reduced the dry weight in red clover nodules compared to the non-defoliated control ($P<0.05$; Figs. 3.1H).

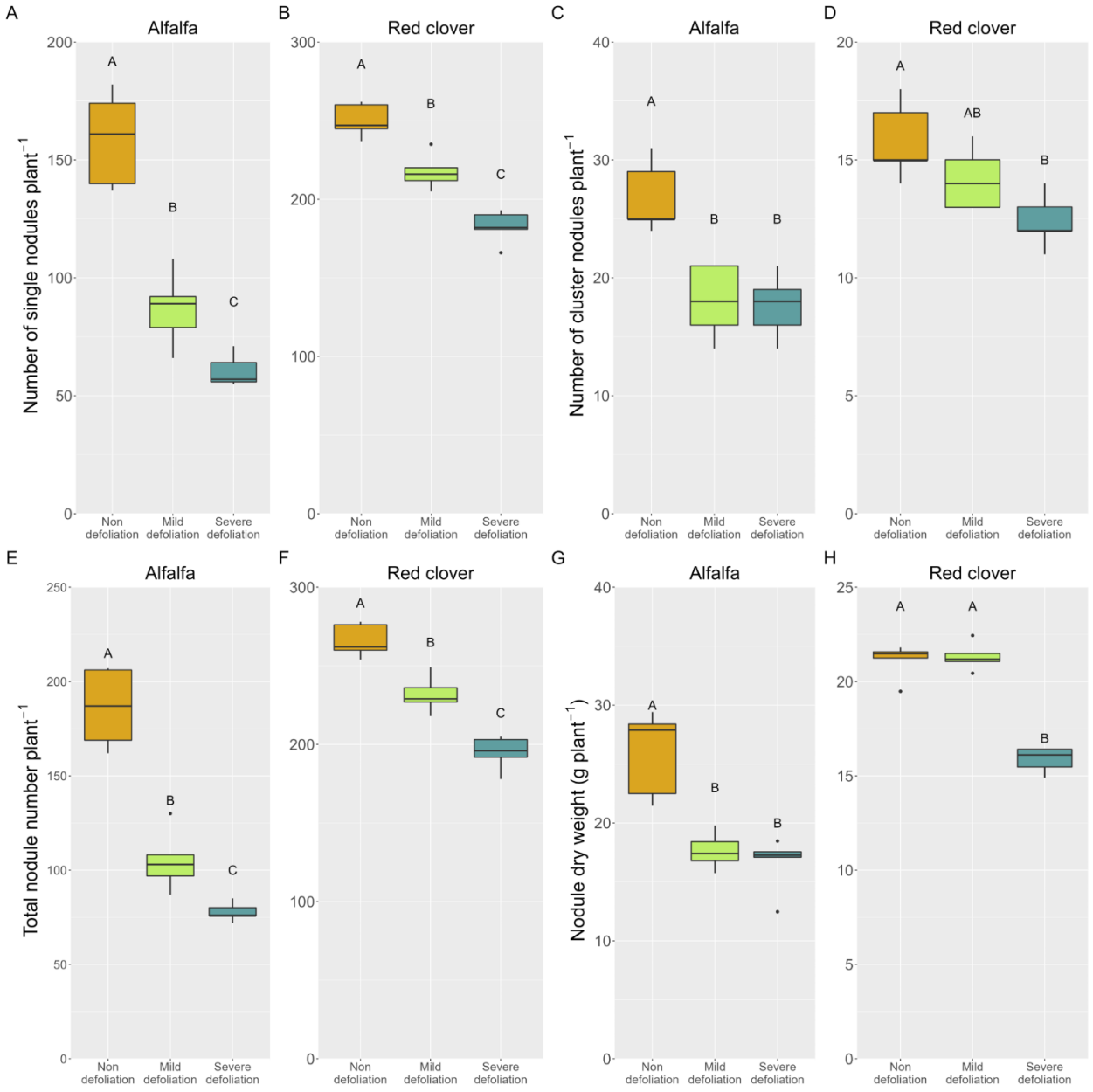


Figure 3.1. The effect of mild (50%) and severe defoliation (100%) on nodulation parameters in alfalfa and red clover compared to the non-defoliation control: (A-B) number of single nodules per plant; (C-D) number of cluster nodules per plant; (E-F) total nodule number per plant; (G-H) nodule dry weight per plant. Different letters indicate significant differences between defoliation treatments at $p < 0.05$. Each treatment comprised five replicates.

3.3.2 Effect of defoliation on plant biomass and root traits

Total shoot dry weight for both mild and severe defoliation treatments was calculated by adding the dry weights of the first cut (defoliation) and final cut after the 4-week recovery period, while shoot weight for the non-defoliated control was obtained based only on shoots harvested at the end of the growing period. Mild defoliation increased alfalfa total shoot biomass compared to the non-defoliation control ($P<0.05$; Fig. 3.2A), while total shoot dry weight of plants severely defoliated was not significantly different from control and mildly defoliated plants. In contrast, red clover plants subjected to mild defoliation significantly increased the total shoot biomass compared to the severely defoliated plants ($P<0.05$; Fig. 3.2B). Severe defoliation significantly reduced the root dry weight in both alfalfa and red clover compared to the non-defoliation control ($P<0.01$; Figs. 3.2C, D). There was no significant effect of mild defoliation on alfalfa root length, in contrast, severe defoliation significantly reduced the root length compared to the control ($P<0.0001$; Fig. 3.2E). In red clover, root length was significantly reduced under both defoliation treatments compared to the control ($P<0.0001$; Fig. 3.2F). Defoliation stress significantly reduced the root surface area in both species compared to undefoliated control plants ($P<0.0001$; Figs. 3.2G, H). Root volume in alfalfa was significantly reduced only under severe defoliation stress compared to the control ($P<0.0001$; Fig. 3.2I), while in red clover, both defoliation treatments significantly reduced the root volume ($P<0.0001$; Fig. 3.2J). Lastly, severe defoliation significantly decreased average root diameter in alfalfa ($P<0.0001$; Fig. 3.2K) and red clover ($P<0.0001$; Fig. 3.2L) compared to non-defoliation control.

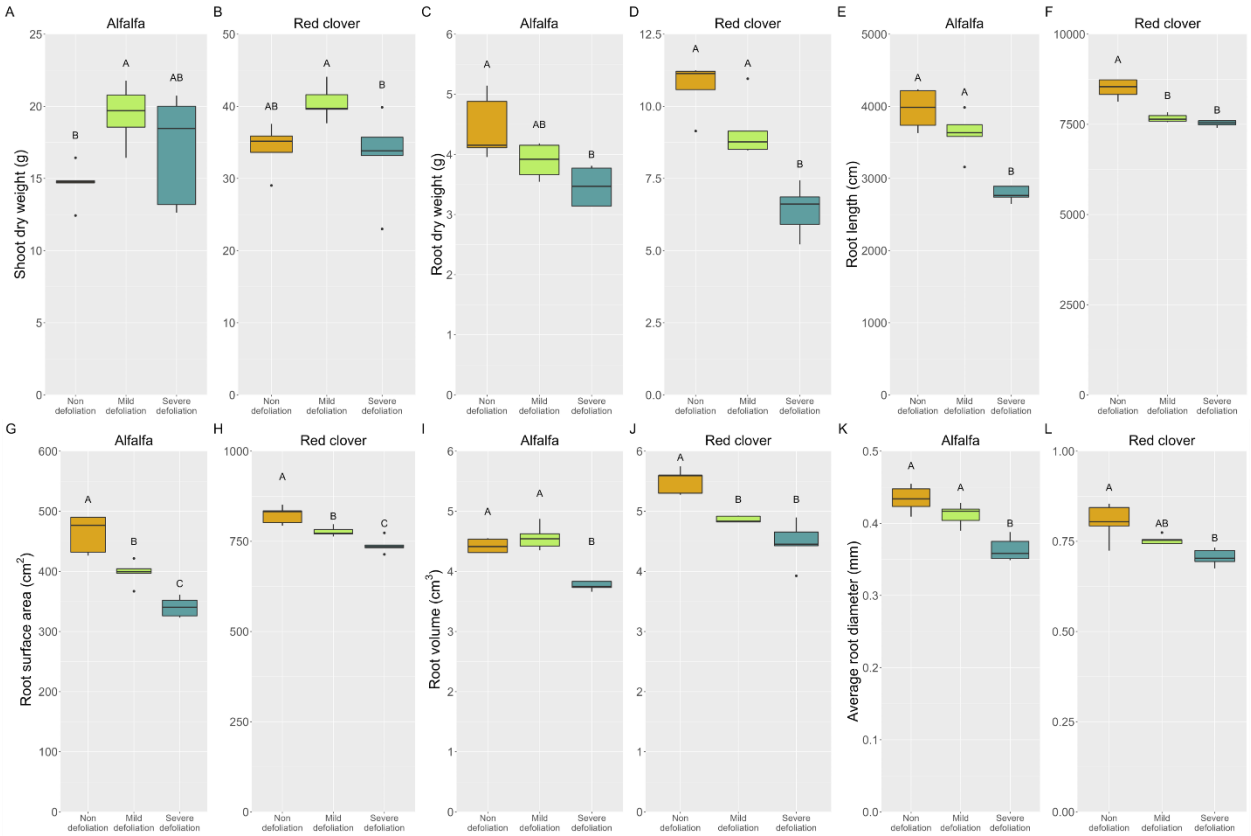


Figure 3.2. The effect of mild (50%) and severe defoliation (100%) on plant biomass and root traits of alfalfa and red clover compared to the non-defoliation control. (A-B) Shoot dry weight; (C-D) Root dry weight; (E-F) Total root length; (G-H) Root surface area; (I-J) Root volume; (K-L) Average root diameter. Different letters indicate significant differences between defoliation treatments at $p < 0.05$. Each treatment comprised five replicates.

3.3.3 Effect of defoliation on shoot nitrogen and nitrogen fixation

The average %Ndfa in alfalfa and red clover shoot samples from the defoliation treatment (first cut) was 50% ± 3.0 and 65% ± 2.4, respectively (Fig. B.1). Defoliation treatments did not affect the shoot N concentration of the final harvest compared to the non-defoliated control in both plant species (Figs. 3.3A, B). The total shoot N content of plants subjected to defoliation treatments was calculated by adding data from the defoliated and the final shoot N content values. The shoot total N content was higher under mild defoliation compared to the control in alfalfa plants ($P<0.05$; Fig. 3.3C). In contrast, red clover total shoot N content was not influenced by the defoliation

treatments compared to the control (Fig. 3.3D). While there was no significant effect of defoliation stress on alfalfa nitrogen fixation capacity (%Ndfa) in the final shoot (Fig. 3.3E), nitrogen fixation was reduced significantly in red clover following the severe defoliation (P=0.01; Fig. 3.3F). Finally, the total shoot N fixed per plant was obtained by combining defoliated and final shoot N fixation values. The total shoot N fixed was significantly higher under severe defoliation compared to the non-defoliated control in alfalfa (P<0.05; Fig. 3.3G), while no there was significant difference in the total N fixed in red clover (Fig. 3.3H).

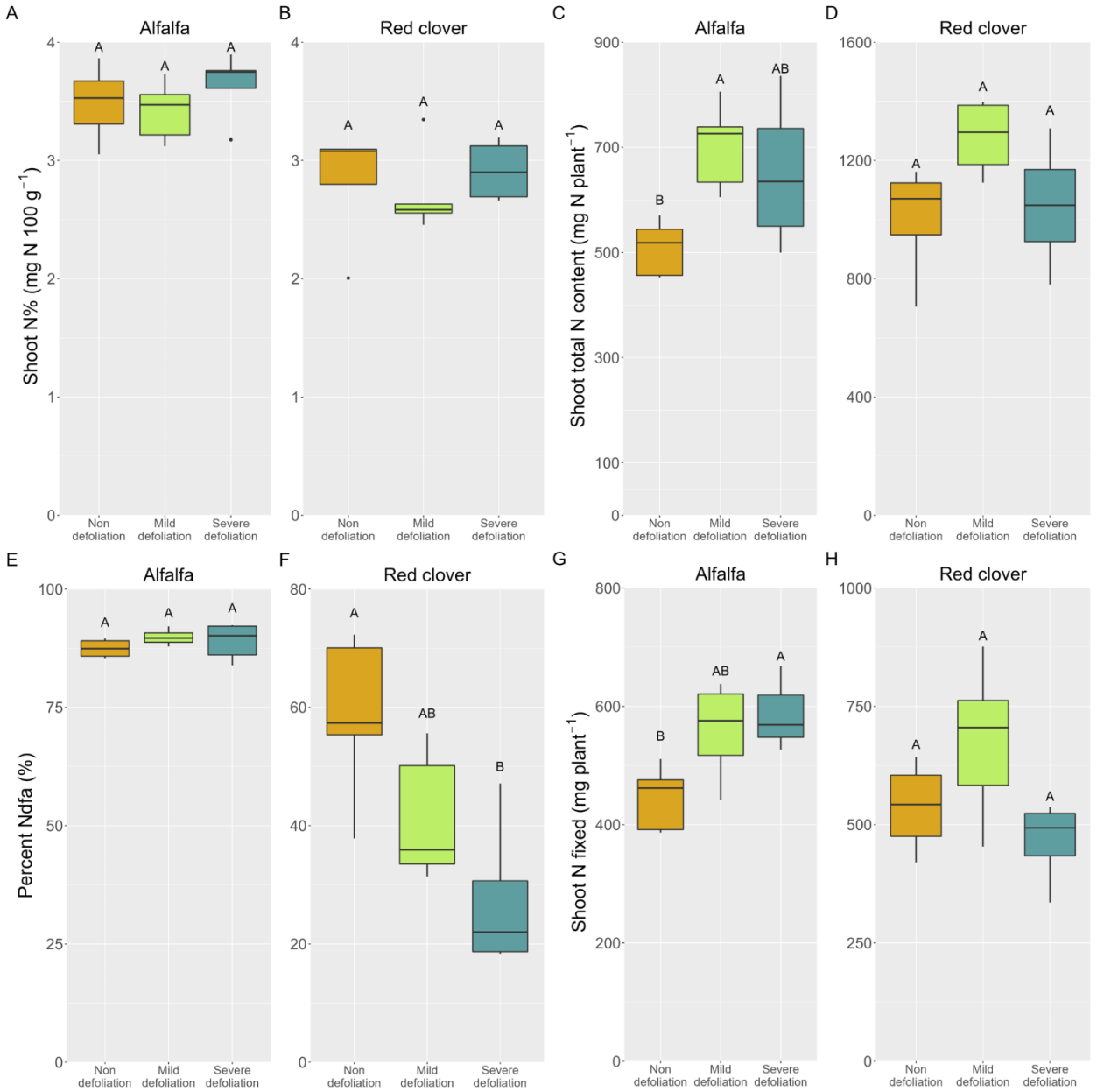


Figure 3.3. The effects of mild (50%) and severe defoliation (100%) on shoot nitrogen and nitrogen fixation of alfalfa and red clover compared to non-defoliation control. (A-B) Shoot nitrogen concentration; (C-D) Shoot total N content per plant; (E-F) Percent (%) nitrogen derived from the atmosphere (%Ndfa); (G-H) Total shoot nitrogen fixed per plant. Different letters indicate significant differences between defoliation treatments at $p < 0.05$. Each treatment comprised five replicates.

3.3.4 Effect of defoliation on soil available nitrogen and soil enzyme activity

The soil available NO_3^- and NH_4^+ were measured using the PRS probes and together represent soil available N. Nitrate was the predominant available N form in the soils under all treatments (Fig. B.2). Soil available N was not significantly different between different defoliation treatments in alfalfa following one week of defoliation and four weeks of recovery (Figs. 3.4A, C). In contrast, the soil available N was significantly higher in red clover following one week of severe defoliation ($P<0.05$, Fig. 3.4B) and four weeks of recovery ($P<0.001$; Fig. 3.4D) compared to mild defoliation and non-defoliation control. Defoliation increased soil C and phosphate extracellular enzyme activities following in both plant species. The β-1, 4-glucosidase ($P<0.001$; Figs. 3.4E, F) and β-D-cellobiosidase ($P<0.001$; Figs. 3.4G, H) activities were significantly higher under severe defoliation compared to non-defoliation control. Phosphatase activity in both plant species was significantly higher under moderate and severe defoliation in alfalfa ($P<0.01$; Fig. 3.4I) and red clover ($P<0.001$; Fig. 3.4J). Lastly, N-acetyl-β glucosaminidase activity in both legumes was not affected by defoliation treatment (Figs. 3.4K, L).

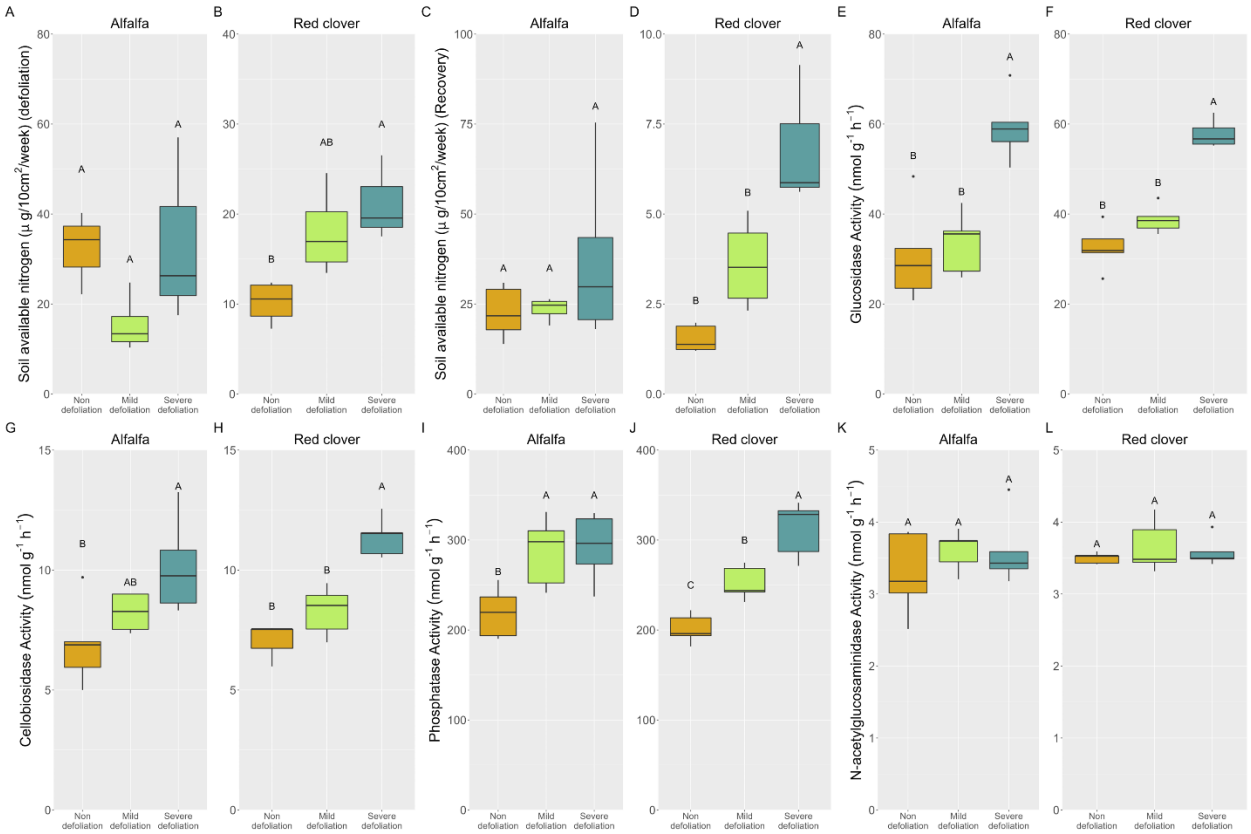
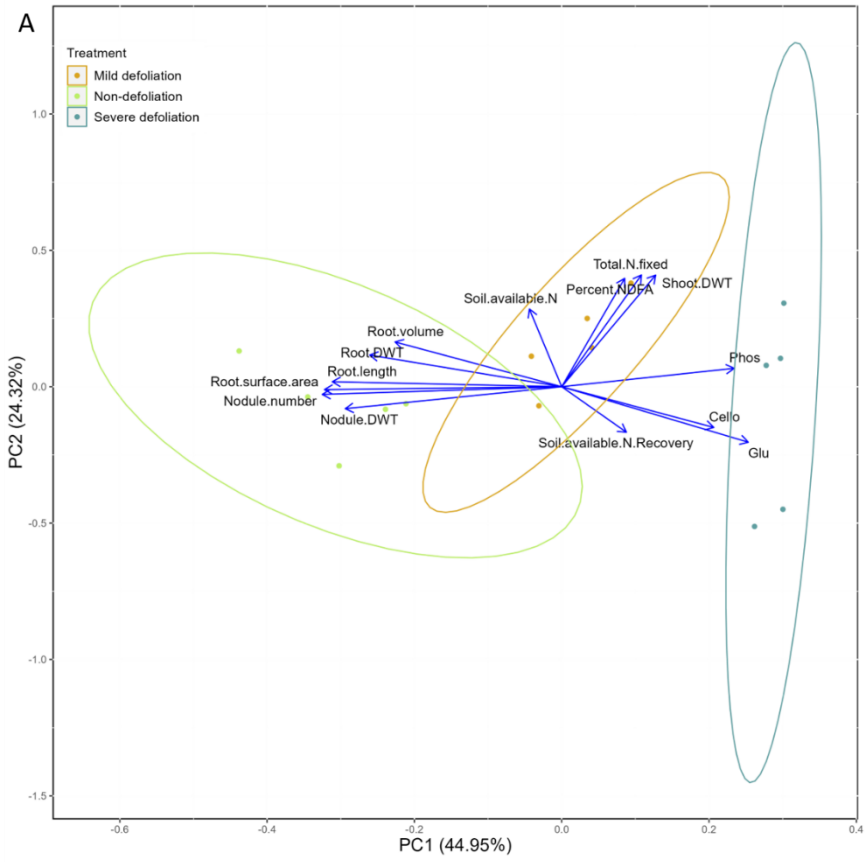


Figure 3.4. The effects of mild (50%) and severe defoliation (100%) on soil available nitrogen and extracellular enzyme activities compared to non-defoliation control. (A-B) total soil available nitrogen following one week of defoliation; (C-D) total soil available nitrogen following four weeks of recovery; (E-F) β-1, 4-glucosidase (Glu) activity; (G-H) β-D-cellobiosidase (Cello) activity; (I-J) acid phosphatase (Phos) activity; (K-L) N-acetyl-β-glucosaminidase (NAG) activity. Different letters indicate significant differences between defoliation treatments at $p < 0.05$. Each treatment comprised five replicates.

3.3.5 Principal component analysis of different plant and soil parameter

In the PCA biplot, the two components accounted for 69.3% of the total variance in alfalfa (Fig. 3.5A), while 81.3% was accounted for red clover in the 14 attributes considered (Fig. 3.5B). For both plant species, individual plants clustered together based on defoliation treatment, and this pattern is especially apparent for alfalfa. In red clover, severe defoliation-treated individuals clustered separately from the mild and non-defoliated treatments (Fig. 3.5B). The variables in

alfalfa that were positively correlated with non-defoliation were nodule number, nodule dry weight, root dry weight, root length, root volume, and root surface area (Fig. 3.5A). The shoot dry weight, %NDFA, and total N fixed positively correlated with mild defoliation, while Cello, Glu, Phos, and soil available N positively correlated with severe defoliation (Fig. 3.5A). The variables in red clover that positively correlated with non-defoliation and mild defoliation were nodule number, nodule dry weight, root and shoot dry weight, root length, root volume, root surface area, %NDFA, and total N fixed (Fig. 3.5B), while soil available N, Cello, Glu and Phos were positively correlated with severe defoliation (Fig. 3.5B).


A
Treatment
Mild defoliation
Non-defoliation
Severe defoliation
Total.N.fixed
Percent.NDFA
Shoot.DWT
Soil.available.N
Root.volume
Root.DWT
Root.length
Root.surface.area
Nodule.number
Nodule.DWT
Phos
Cello
Glu
Soil.available.N.Recovery
PC2 (24.32%)
PC1 (44.95%)

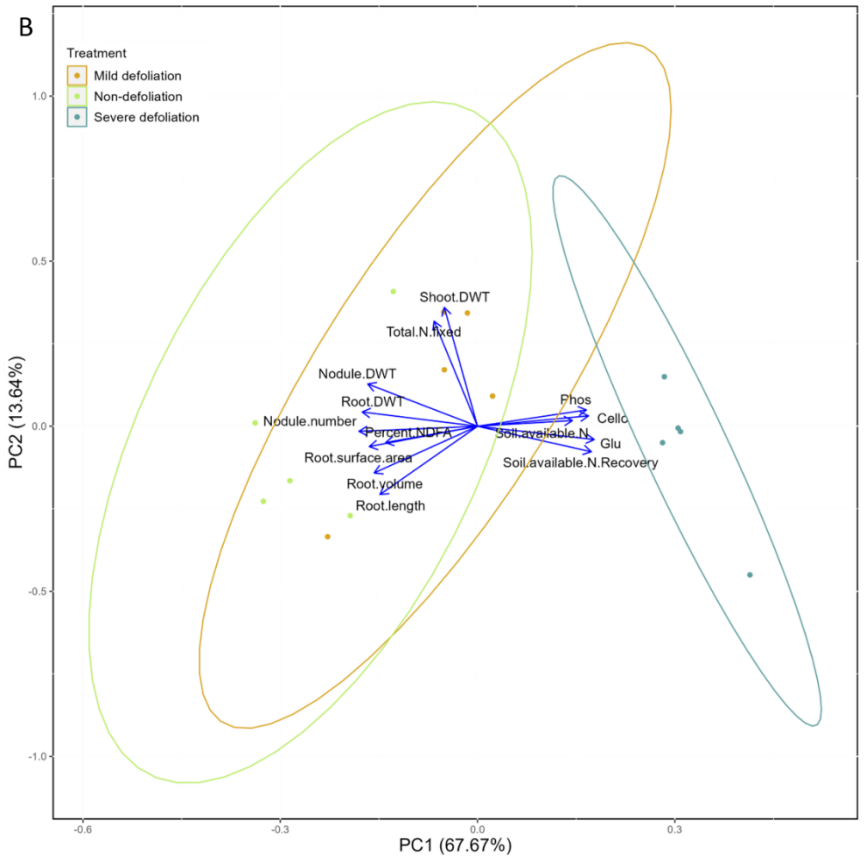

B
Treatment
Mild defoliation
Non-defoliation
Severe defoliation
Shoot.DWT
Total.N.fixed
Nodule.DWT
Root.DWT
Nodule.number
Percent.NDFA
Root.surface.area
Root.volume
Root.length
Phos
Cello
Soil.available.N
Glu
Soil.available.N.Recovery
PC2 (13.64%)
PC1 (67.67%)

Figure 3.5. Principal component analysis of nodulation parameters (nodule number, dry weight), shoot and root biomass, root traits (length, volume, surface area), percent nitrogen derived from the atmosphere (%Ndfa), total nitrogen fixed, soil available nitrogen, soil available nitrogen at recovery, and extracellular enzyme activity: Cello, Glu, Phos of alfalfa (A) and red clover (B) plants under non-defoliation (0%), mild defoliation (50%), and severe defoliation (100%). DWT, dry weight; Cello, Cellobiosidase; Glu, Glucosidase; Phos, Acid Phosphatase.

3.3.6 Effect of defoliation on soil microbial community structure

Actinobacteria was predominantly abundant in both bulk and rhizosphere under both plant species at the phylum level, followed by Proteobacteria, Firmicutes, and Acidobacteria (Figs. 3.6A, B). Based on alpha-diversity analysis, Shannon diversity was not significantly different among different defoliation treatments of alfalfa and red clover in both rhizosphere and bulk soil (Figs. 3.7A-D). However, the Shannon diversity index under severely defoliated alfalfa plants, although not statistically significant, was marginally higher in both bulk and rhizosphere compared to the non-defoliation control (Figs. 3.7 A, C). Furthermore, we noted that there was no shift in the bacterial community in either plant influenced by defoliation, as analyzed through permutational analysis of variance (PERMANOVA) with Bray-Curtis dissimilarity matrix (Fig. B.3). The bacterial community was distinct from rhizosphere to bulk soil in both legume plants ($P<0.01$; Fig. B.3).

The influence of defoliation intensity on the relative abundance of some taxonomic groups was more pronounced in alfalfa rhizosphere samples compared to alfalfa bulk and both red clover bulk and rhizosphere samples where we did not observe any significant differences in any taxa (Figs. 3.7E, F). The relative abundance of Gp3 from the Acidobacteria subgroups was significantly higher in the rhizosphere of severe defoliation treated plants compared to non-defoliation and mild defoliation treatments ($P<0.01$; Figs. 3.7E).

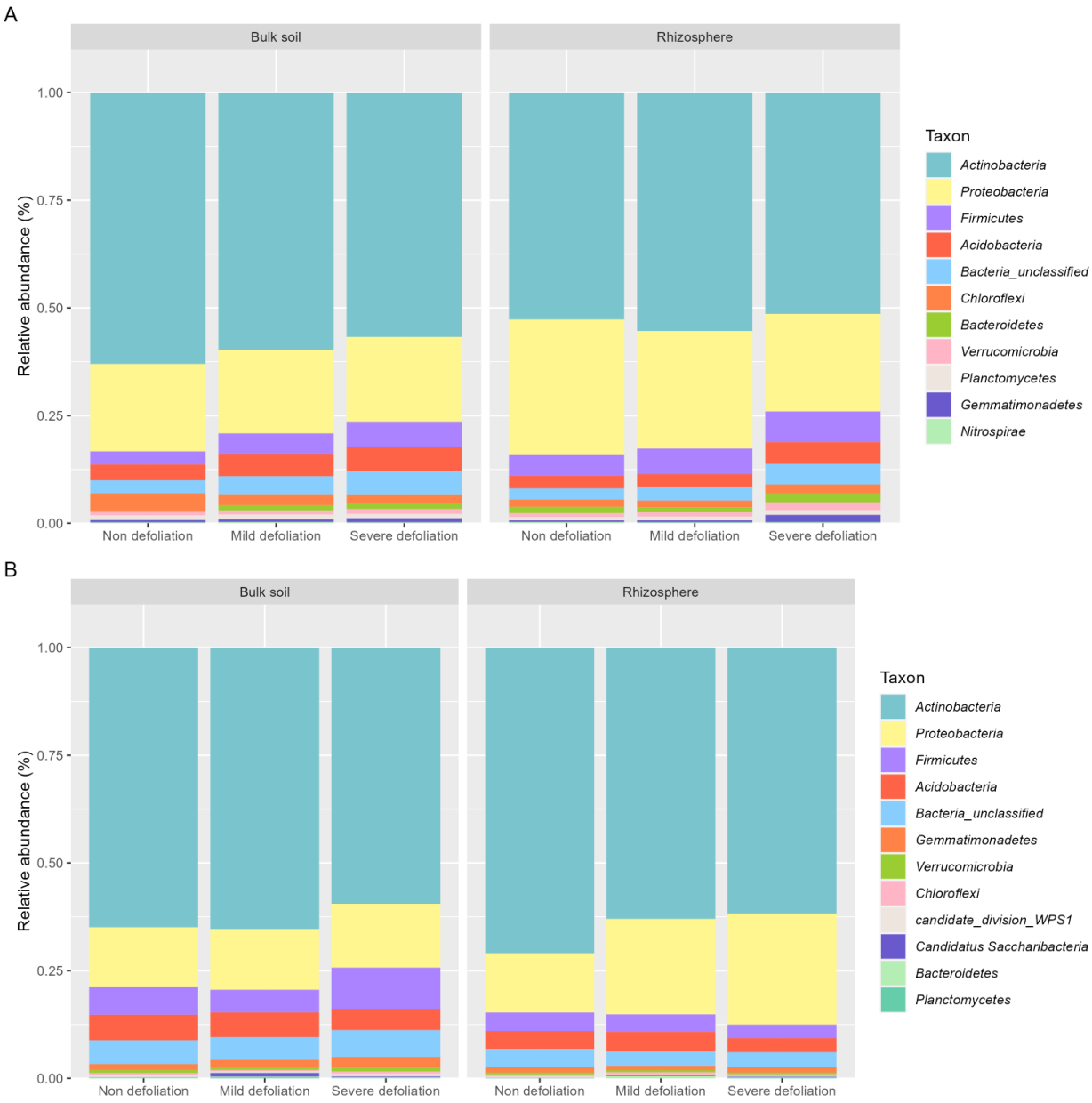


Figure 3.6. Distribution and abundance analysis of bacterial communities in bulk and rhizosphere soil under alfalfa and red clover following different simulated defoliation intensities. (A) Alfalfa phyla relative abundance; (B) Red clover phyla relative abundance. Legend shows phylum phylogenetic level.

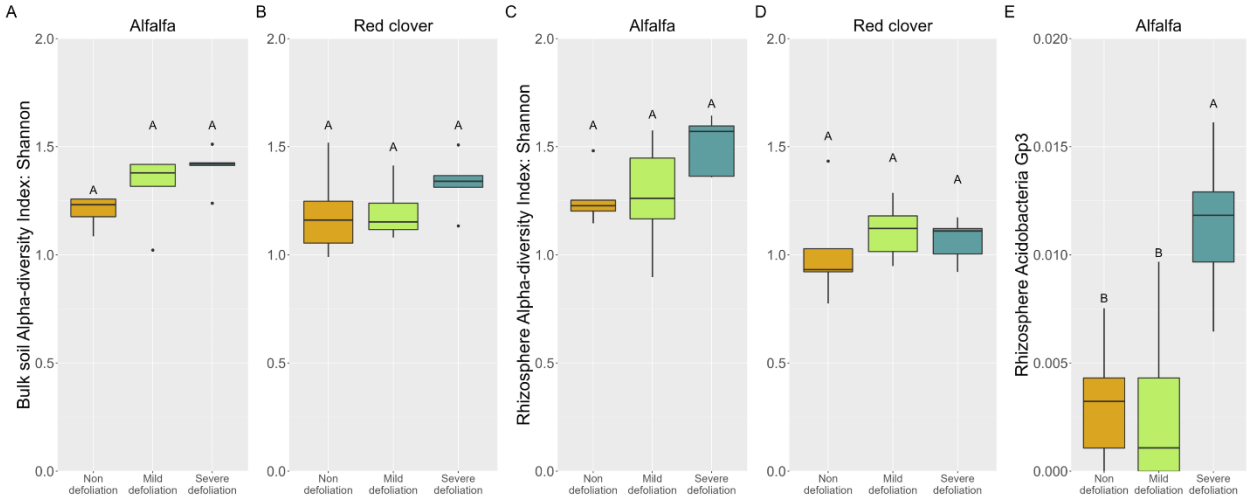


Figure 3.7. Microbiome analysis of alfalfa and red clover soil and rhizosphere samples following mild (50%) and severe defoliation (100%) compared to non-defoliation control. (A-D) Shannon diversity indices of alfalfa and red clover bulk (A-B) and rhizosphere (C-D) samples. Rhizosphere relative abundances: Class Acidobacteria Gp3 (E). Different letters indicate significant differences between soil moisture treatments at $p < 0.05$.

3.4 Discussion

Alfalfa (*Medicago sativa*) and red clover (*Trifolium pratense*) are two important forage legumes in pasture production (Sleugh et al., 2000; Sanderson et al., 2013) and have been reported to fix significant amounts of N_2 (~78 to 222 kg N ha^{-1} yr^{-1}) (Fageria, 2014; De Haan et al., 2017). Defoliation is known to influence legume SNF by removing the source of photosynthates needed for the process, affecting plant growth and yield production (Rimi et al., 2014; Xu et al., 2009) and inducing changes in belowground dynamics between plants and soil (Marriott and Haystead, 1990; Chesney and Nygren, 2002; Ayres et al., 2007). Interestingly, the present study found that defoliation negatively affected nodulation and other root phenotypic traits, had positive effects on C and phosphate extracellular enzyme activities, and had a diverse influence on the shoot growth, SNF, and soil available nitrogen depending on the forage legume species. Hence, observing the response of plant phenological traits and SNF to defoliation stress will help in understanding the relation of the plant system response to soil health.

Defoliation induces nodule senescence (Chesney and Nygren, 2002), leading to the decreased nodule number and nodule dry weight, and our data corroborate with these previous observations (Aranjuelo et al., 2015; Chesney and Nygren, 2002). Defoliation stress triggers various plant physiological and biochemical responses, primarily due to the loss of photosynthetic tissues, affecting existing nodules, including: (1) reduced nitrogenase activity (Cralle and Heichel 1981: Denison et al., 1992), (2) decreased O_2 permeability which causes inactivation of nitrogenase (Denison et al., 1992; Nygren et al., 2000; Sulieman and Tran, 2016), and (3) increased activity of protease and nitrate reductase inducing senescence (Vance et al., 1979). Furthermore, the removal of leaves. which are the main N sinks, through defoliation leads to the accumulation of fixed N in roots and nodules and its eventual release into the soil due to tissue senescence (Ayres et al., 2004, 2007; Carrillo et al., 2011), increasing the pool of available soil N. This study found the same trend of increased available soil N following defoliation, suggesting that defoliation enhances soil N pool days or even weeks following the treatment (Ayres et al., 2007; Carrillo et al., 2011; Hamilton et al., 2008).

The activity of C and P cycling enzymes was increased with defoliation, which corroborates with field studies examining the effects of defoliation (Hewins et al. 2016). The increase in C cycling enzymes, cellobiosidase and glucosidase could be attributed to the increase in root exudation and root decomposition induced by defoliation (Belsky 1986). As observed in our PCA analysis, EEAs were positively correlated with soil available N, suggesting an increase in root exudation following defoliation that changes microbial activity in the soil (Kuzyakov and Xu, 2013). Extracellular enzyme activity is correlated with root biomass due to the amount of nutrients that can be rhizodeposited into the soil (Kanté et al. 2021). Furthermore, both root growth and death can increase rhizodeposition through the sloughing-off of root border cells and tissue senescence, respectively (Fustec et al. 2010). Similar to C cycling enzymes, phosphatase increased with defoliation, which suggests increased organic P in exudates and plant tissue. Previous research showed that grazed plants had higher P tissue concentration, suggesting that P intake was enhanced following foliar damage (Li et al., 2010). It is likely that increased plant demand for P induced microbial activity to improve P cycling enzyme activities to match the P demand from both plants and microbes (Hewins et al. 2016). Lastly, NAG activity was not increased despite the increased available N in the soil. This can be due to a lack of enrichment of the bacterial population specialized in N cycling from the subgroup Gp3 of Acidobacteria (Kang et al., 2021; Ward et al.,

2009; Belova et al., 2018) or due to the rapid mineralization of N and the temporal limitation of enzyme assays (Bai et al., 2021; Kang et al., 2021; Hewins et al., 2016). In the last case, either an *In situ* enzyme assay protocol or conducting sampling for EEA immediately following defoliation can reveal more information on the effect of defoliation on soil enzyme activity. Additionally, defoliation may have triggered a rapid release of fixed nitrogen through nodules and roots (Thilakarathna et al., 2019), causing enrichment of available nitrogen in the soil that plants and microbes can readily use.

Our study showed that mild defoliation significantly increased the total plant biomass. After being defoliated, the remaining leaf biomass and root reserves play a crucial role in allocating organic reserves for regrowth (Meuriot et al., 2004). Recovery from defoliation necessitates the mobilization of both C and N resources, and alfalfa was shown to rely heavily on endogenous N pools to support regrowth (Ourry et al., 1994; Meuriot et al., 2005; Teixeira et al., 2007). The results from the present study also showed increased shoot N content in alfalfa following mild defoliation treatment and no changes in N fixation capacity (%Ndfa) under different defoliation treatments, suggesting movement of N from belowground tissues. On the other hand, root dry weight of alfalfa and red clover was reduced, and their root architecture altered following defoliation stress, possibly due to the reallocation of C resources for shoot regrowth of the remaining above-ground tissues or the loss of resources likely through increased rhizodeposition and tissue senescence (Davidson and Milthorpe, 1966; Richards 1993).

Many studies have pointed out that plants exert selection pressures on soil microbes, directly and indirectly through their belowground tissues (Hamilton and Frank 2001; Dennis et al. 2010; Huang et al. 2014; Shi et al. 2015). This study investigated how defoliation of two forage legumes influenced the distinct differences observed in the beta diversity of the bacterial community structure between bulk and rhizosphere environments. Plants respond to defoliation by changing the source-sink dynamics between their aboveground and belowground tissues, especially in relation to photosynthates (Keoghan 1982; Baysdorfer and Bassham 1985; Black et al. 2009; Proulx and Naeve 2009). The effects of defoliation stress on plant growth and the consequential influence on C and N rhizodeposition can subsequently affect the root-associated bacterial communities, as indicated by our study.

Many organic byproducts of photosynthesis are translocated and released by the roots and root organs through rhizodeposition (Dennis et al. 2010). Root-derived C compounds, along with

other N and P containing compounds, are the primary constituents of rhizodeposits (Hu et al., 2018; Ankati and Podile, 2019). As these nutrients are growth-limiting, they are strong determinants of the structure of soil microbes (Marilley et al., 1998; Shi et al., 2015; Dennis et al., 2010). Defoliation plays a role in affecting the amount of rhizodeposits transferred into the rhizosphere and surrounding soil. Studies have shown that mild defoliation induces the reallocation of resources to the remaining crown for regrowth. On the other hand, severe foliar damage promotes the allocation of the residual C to the roots and its eventual loss through rhizodeposition and tissue senescence (Aranjuelo et al., 2015; Hamilton et al., 2008; Wilson et al., 2018), increasing the soil resource pool.

Resource availability through rhizodeposits is a major driver of soil microbial assembly (Corel et al. 2016). The evident separation of bulk soil bacterial communities from the rhizosphere in red clover suggests that the rhizodeposits drive the recruitment of a subset of bacteria from the bulk soil into the rhizosphere (Finkel et al. 2017), and that this interaction can be influenced by a multitude of host-specific and environmental conditions (Gaiero et al. 2013; Fitzpatrick et al. 2018). Here, we observed a trend that bacterial diversity in bulk soil and rhizosphere in both legume plants was higher following defoliation, although not statistically significant. The positive effect of defoliation intensity linked to resource availability in the rhizosphere and surrounding soil environment (Hamilton and Frank 2001; Kuzyakov et al. 2007; Ma et al. 2018; Tian et al., 2019). The increased abundance of Acidobacteria subgroup Gp3 in the rhizosphere following severe defoliation corroborates with the findings of Ma et al. (2018). This is associated with their oligotrophic nature, where these microbes are known to thrive in soil ecosystems under low C availability (Fierer et al. 2007; Xun et al. 2016). Members of the Acidobacteria phylum are noted to have high plasticity and thus are physiologically diverse (Huber et al., 2022). Also, bacterial species under in the Acidobacteria are crucial players in N cycling (Ward et al., 2009). Defoliation's impact on this microbial taxon’s abundance is linked to the preferential allocation of C-rich photosynthates to above-ground tissues to sustain foliar growth or loss of C resources in the tissues through deposition and senescence (Aranjuelo et al., 2015; Xu et al. 2013; Moot et al. 2021), limiting provisions to below-ground tissue, which promotes oligotrophs in the rhizosphere.

This research found some positive and negative effects of mild and severe defoliation on aboveground (e.g., shoot growth, shoot nitrogen, SNF) and belowground (e.g., nodulation, root phenotypes, soil enzyme activities, soil N availability, soil microbiome) parameters of two

important forage legumes. Generally, defoliation had negative effects on nodulation, root growth in both legumes, and SNF of red clover. Soil available N following defoliation and after recovery was significantly increased in red clover. The extracellular enzyme assay showed that defoliation increased the activities of β-1, 4-glucosidase, β-D-cellobiosidase and phosphatase enzyme in both forage legumes, suggesting an increased C and P source exudation into the soil. Microbiome analysis showed differential responses of the two forage plant species under defoliation stress conditions. The alpha- and beta-diversity of soil bacteria were not significantly affected by defoliation stress. However, we observed the enrichment in the relative abundance of Acidobacteria Gp3 in the alfalfa rhizosphere. The overall results indicate that defoliation causes negative effects on nodulation and root growth, while positively influencing soil N availability, likely through increased tissue senescence and exudation, total shoot biomass and specific soil microbial taxa.

References

Adentuji AT, Ncube B, Mulidzi R, Lewu FB (2020) Potential use of soil enzymes as soil quality indicators in agriculture. Front Soil Environ Micro 6: 58-62.

Ankati S, Podile AR (2019) Metabolites in the root exudates of groundnut change during interaction. J Plant Physiol 243: 153057.

Aranjuelo I, Molero G, Erice G, Aldasoro J, Arrese-Igor C, Nogués S (2015) Effect of shoot removal on remobilization of carbon and nitrogen during regrowth of nitrogen-fixing alfalfa. Physiology Plant 153:91-104.

Atkins CA, Smith PMC (2007) Translocation in legumes: assimilates, nutrients, and signaling molecules. Plant Physiol 144: 550-561.

Atkins CA, Pate JS, Sharkey PJ (1982) Asparagine metabolism-key to the nitrogen nutrition of developing legume seeds. Plant Physiol 56: 807-812

Ayres E, Heath J, Possell M, Black HIJ, Kerstiens G, Bardgett RD (2004) Tree physiological responses to above-ground herbivory directly modify below-ground processes of soil carbon and nitrogen cycling. Ecol Lett 7: 469-479.

Ayres E, Dromph KM, Cook R, Ostle N, Bardgett RD (2007) The influence of below-ground herbivory and defoliation of a legume on nitrogen transfer to neighbouring plants. Funct Ecol 21: 256-263.

Bai X, Dippold MA, An S, Wang B, Zhang H, Loeppmann S (2021) Extracellular enzyme activity and stoichiometry: the effect of soil microbial element limitation during leaf litter decomposition. Ecol Indic 121: 107200.

Bausenwein U, Gattinger A, Langer U, Embacher A, Hartmann H-P, Sommer M, Munch JC, Schloter M (2008) Exploring soil microbial communities and soil organic matter variability and interactions in arable soils under minimum tillage practice. Appl Soil Ecol 40: 67-77.

Baysdorfer C, Bassham JA (1985) Photosynthate supply and utilization in alfalfa: a developmental shift from a source to a sink limitation of photosynthesis. Plant Physiol 77: 313-317.

Belova SE, Ravin NV, Pankratov TA, Rakitin AL, Ivanova AA, Beletsky AV, Mardanov AV, Sinninghe Damsté JS, Dedysh SN (2018) Hydrolytic capabilities as a key to

environmental success: chitinolytic and cellulolytic *Acidobacteria* from acidic sub-arctic soils and boreal peatlands. Front Microbiol 19: 2775.

Belsky AJ (1986) Does Herbivory Benefit Plants? A review of the evidence. Amer Soc Nat 12: 870-892.

Berg G, Rybakova D, Grube M, Köberl M (2016) The plant microbiome explored: implications for experimental botany. J Exp Bot 67: 995-1002.

Black AS, Laidlaw AS, Moot DJ, O'Kiely P (2009) Comparative growth and management of white and red clovers. IJAFR 48: 149-166.

Bowles TM, Acosta-Martínez V, Calderón F, Jackson LE (2014) Soil enzyme activities, microbial communities, and carbon and nitrogen availability in organic agroecosystem across an intensively-managed agricultural landscape. Soil Biol Biochem 68: 252:262.

Carrillo Y, Jordan CF, Jacobsen KL, Mitchell KG, Raber P (2011) Shoot pruning of a hedgerow perennial legume alters the availability and temporal dynamics of root-derived nitrogen in a subtropical setting. Plant Soil 345: 59-68.

Carter AM, Tegeder M (2016) Increasing nitrogen fixation and seed development in soybean requires complex adjustments of nodule nitrogen metabolism and partitioning processes. Curr Biol 26: 2044-2051

Cavaglieri L, Orlando J, Etcheverry M (2009) Rhizosphere microbial community structure at different maize plant growth stages and root locations. Microbiol Res 164: 391-399.

Chaparro JM, Badri DV, Bakker MG, Sugiyama A, Manter DK, Vivanco JM (2013) Root exudation of phytochemicals in Arabidopsis Follows Specific Patterns That Are Developmentally Programmed and Correlate with Soil Microbial Functions. PLoS ONE 8, e55731.

Chaparro JM, Badri DV, Vivanco JM (2014) Rhizosphere microbiome assemblage is affected by plant development. ISME J 8: 790-803. https://doi.org/10.1038/ismej.2013.196Chong J, Liu P, Zhou G, Xia J (2020) Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. Nat Protoc 15: 799-821.

Chesney P, Nygren P (2002) Fine root and nodule dynamics of *Erythrina poeppigiana* in an alley cropping system in Costa Rica. Agrifor Syst 56: 259-269.

Chong J, Liu P, Zhou G, Xia J (2020) Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. Nat Protoc 15: 799-821.

Christie BR, Bennett RJ (1984) OAC Minto Alfafla. Can J Plant Sci 64: 419-421.

Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, Brown CT, Porras-Alfaro A, Kuske CR, Tiedje JM (2014) Ribosomal database project: data and tools for high throughput rRNA analysis. Nucleic Acid Res 42: D633-D642.

Corel E, Lopez P, Méheust R, Bapteste E (2016) Network-thinking: graphs to analyze microbial complexity and evolution. Trends in Micro 24: 224-237.

Cralle HT, Heichel GH (1981) Nitrogen fixation and vegetative regrowth of alfalfa and birdsfoot trefoil after successive harvests or floral debudding. Plant Physiol 67: 898-905.

Davidson JL, Milthorpe FL (1996) The effect of defoliation on the carbon balance in *Dactylis glomerata.* Annals Bot 8: 185-198.

De Haan RL, Schuiteman MA, Vos RJ (2017) Residual soil nitrate content and profitability of five cropping systems in northwest Iowa. PLoS ONE 12: e0171994.

del Castillo LD, Hunt S, Layzell DB (1994) The role of oxygen in the regulation of nitrogenase activity in drought-stressed soybean nodules. Plant Physiol 106: 949-955.

Denison RF, Hunt S, Layzell DB (1992) Nitrogenase activity, nodule respiration, and O_2 permeability following detopping of alfalfa and birdsfoot trefoil. Plant Physiol 98: 894-900.

Dennis PG, Miller AJ, Hirsch PR (2010) Are root exudates more important than other sources of rhizodeposits in structuring rhizosphere bacterial communities. FEMS Micro Ecol 72: 313-327.

Fageria NK (2014) Nitrogen harvest index and its association with crop yields. J Plant Nutr 37:6, 795-810.

Fierer N, Bradford MA, Jackson RB (2007) Toward an ecological classification of soil bacteria. Ecol 88: 1354-1364.

Finkel OM, Castrillo G, Paredes SH, González IS, Dangl JL (2017) Understanding and exploiting plant beneficial microbes. Curr Opin Plant Biol 38: 155-163.

Kuzyakov Y, Xu X (2013) Competition between roots and microorganisms for nitrogen: mechanisms and ecological relevance. New Phytol 198: 656-669

Fitzpatrick CR, Copeland J, Wang PW, Guttman DS, Kotanen PM, Johnson MTJ (2018) Assembly and ecological function of the root microbiome across angiosperm plant species. PNAS 115: E1157-E1165.

Fustec J, Lesuffleur F,Mahieu S, Cliquet J-B (2010) Nitrogen rhizodeposition of legumes. A review. Agron Sustain Dev 30: 57-66.

Gaiero JR, McCall CA, Thompson KA, Day NJ, Best AS, Dunfield KE (2013) Inside the root microbiome: bacterial root endophytes and plant growth promotion. Am J Bot 100: 1738-1750.

German DP, Weintraub MN, Grandy AS, Lauber CL, Rinkes ZL, Allison SD (2011) Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. Soil Biol Biochem 43: 1387-1397.

González EM, Larrainzar E, Marino D, Wienkoop S, Gil-Quintana E, Arrese-Igor C (2015) Physiological responses of N2-fixing legumes to water limitation. In: Sulieman S, Tran LS, eds. Legume nitrogen fixation in a changing environment. Springer International Publishing 5-33.

Gordon AJ, Kessler W, Minchin FR (1990) Defoliation-induced stress in nodules of white clover: I. changes in physiological parameters and protein synthesis. J Exp Bot 41: 1245-1253.

Haagenson DM, Cunningham SM, Joern BC, Volenec JJ (2003) Autumn defoliation effects on alfalfa winter survival, root physiology, and gene expression. Crop Sci 43:1340-1348.

Hamilton EW, Frank DA (2001) Can plants stimulate soil microbes and their own nutrient supply? Evidence from a grazing tolerant grass. Ecol 82: 2397-2402.

Hamilton EW, Frank DA, Hinchey PM, Murray TR (2008) Defoliation induces root exudation and triggers positive rhizospheric feedbacks in a temperate grassland. Soil Biol Biochem 40: 2865-2873.

Hewins DB, Fatemi F, Adams B, Carlyle C, Chang SX, Bork EW (2015) Grazing, regional climate and soil biophysical impacts on microbial enzyme activity in grassland soil of Western Canada. Pedobiologia (Jena) 58: 201-209.

Hewins DB, Broadbent T, Carlyle CN, Bork, EW (2016) Extracellular enzyme activity response to defoliation and water addition in two ecosites of the mixed grass prairie. Agri Ecosyst Envi 230: 79-86.

Hu H-W, Wang J-T, Singh BK, Liu Y-R, Chen Y-L, Zhang Y-J, He J-Z (2018) Diversity of herbaceous plants and bacterial communities regulates soil resistome across forest biomes. Environ Microbiol 20: 3186-3200.

Huang X-F, Chaparro JM, Reardon KF, Zhang R, Shen Q, Vivanco JM (2014) Rhizosphere interactions: root exudates, microbes, and microbial communities. Bot 92: 267-275.

Høgh-Jensen H, Schjoerring JK (2001) Rhizodeposition of nitrogen by red clover, white clover and ryegrass leys. Soil Biol Biochem 33: 439-448.

Huber KJ, Vieira S, Sikorski J, Wüst PK, Fösel BU, Gröngröft A, Overmann J (2022) Differential response of acidobacteria to water content, soil type, and land use during an extended drought in African savannah soils. Front Microbiol 13: 750456.

Jacobi A, Katinakis P, Werner D (1994) Artificially induced senescence of soybean root nodules affects different polypeptides and nodulins in the symbiosome membrane compared to physiological ageing. J Plant Physiol 144: 533-540.

Johnson DW, Dijkstra FA, Cheng W (2007) The effects of *Glycine max* and *Helianthus annuus* on nutrient availability in two soils. Soil Biol Biochem 39:2160-2163.

Kang Y, An X, Ma Y, Zeng S, Jiang S, Wu W, Xie C, Wang Z, Dong C, Xu Y, Shen Q (2021) Organic amendments alleviate early defoliation and increase fruit yield by altering assembly patterns and of microbial communities and enzymatic activities in sandy pear (*Pyrus pyrifolia*). AMB Express 11: 164.

Kanté M, Riah-Anglet W, Cliquet J-B, Trinsoutrot-Gattin I (2021) Soil enzyme activity and stoichiometry: linking soil microorganism resource requirement and legume carbon rhizodeposition. Agron 11: 2131.

Keoghan JM (1982) Effects of cutting frequency and height on top-growth of pure lucerne stands. In: Langer, R.H.M. (Ed.), The Lucerne Crop. Wellington, pp. 117–128.

King CA, Purcell LC (2001) Soybean nodule size and relationship to nitrogen fixation response to water deficit. Crop Sci 41: 1099-1107.

Kopture S, Primoli AS, Pimienta MC (2023) Defoliation in perennial plants: predictable and surprising results in *Senna* spp. Plants 12: 587.

Kuzyakov Y, Hill PW, Jones DL (2007) Root exudate components change residue decomposition in a simulated rhizosphere depending on temperature. Plant Soil 290: 293-305.

Li C, Hao X, Willms WD, Zhao M, Han G (2010) Effect of long-term cattle grazing on seasonal nitrogen and phosphorus concentrations in range forage species in the fescue grassland of southwestern Alberta. J Plant Nutr Soil Sci 173: 946-951.

Ling N, Wang T, Kuzyakov Y (2022) Rhizosphere bacteriome structure and functions. Nat Commun 13: 836

Liu J, Chen J, Chen G, Guo J, Li Y (2020) Enzyme stoichiometry indicates the variation of microbial nutrient requirements at different soil depths in subtropical forests. PLoS ONE 15: e0220599.

Liyanage DK, Torkamaneh D, Belzile F, Balasubramanian P, Hill B, Thilakarathna MS (2023) The genotypic variability among short-season soybean cultivars for nitrogen fixation under drought stress. Plants 12: 1004.

Ma B, Cai Y, Bork EW, Chang SX (2018) Defoliation intensity and elevated precipitation effects on microbiome and interactome depend on site type in northern mixed-grass prairie. Soil Biol Biochem 122:163-172.

Macdonald LM, Paterson E, Dawson LA, McDonald AJS (2006) Defoliation and fertiliser influences on the soil microbial community associated with two contrasting *Lolium perenne* cultivars. Soil Biol Biochem 38: 674-682.

Marriott CA, Haystead A (1990) The effect of defoliation on the nitrogen economy of white clover: regrowth and the remobilization of plant organic nitrogen. Annals Bot 66: 465-474.

Marilley L, Vogt G, Blanc M, Aragno M (1998) Bacterial diversity in the bulk soil and rhizosphere fractions of Lolium perenne and Trifolium repens as revealed by PCR restriction analysis of 16S rDNA. Plant and Soil 198: 219–224.

McPherson MR, Wang P, Marsh EL, Mitchell RB, Schachtman DP (2018) Isolation and analysis of microbial communities in soil, rhizosphere, and roots in perennial grass experiments. J Vis Exp 137: e57932.

Meuriot F, Avice JC, Simon JC, Laine P, Decau LM, Ourry A. (2004) Influence of initial organic N reserves and residual leaf area on growth, N uptake, N partitioning and N storage in alfalfa (*Medicago sativa*) during post-cutting regrowth. Annals of Bot 94: 311-321.

Meuriot F, Decau M-L, Bertrand AM, Prud'Homme M-P, Gastal F, Simon J-C et al. (2005) Contribution of initial C and N resereves in *Medicago sativa* recovering from defoliation: impact of cutting height and residual leaf area. Funct Plant Biol 32- 321-334.

Moot DJ, Black AD, Lyons EM, Egan LM, Hofmann RW (2021) Pasture resilience reflects differences in root and shoot responses to defoliation, and water and nitrogen deficit. Resilient Pastures – Grassland Research and Practice Series 17: 71-80.

Nishida H, Suzaki T (2018) Nitrate-mediated control of root nodule symbiosis. Curr Opin Plant Biol 44: 129-136.

Nygren P, Vaillant V, Desfontaines L, Cruz P, Domenach AM (2000) Effects of nitrogen source and defoliation on growth and biological dinitrogen fixation of Gliricidia sepius seedlings. Tree Physiol 20: 33-40.

Ourry A, Kim TH, Boucaud J (1994) Nitrogen reserve mobilization during regrowth of *Medicago sativa* L. Plant Physiol 105: 831-837.

Pirhofer-Walzl K, Rasmussen J, Høgh-JensenH, Eriksen J, Søegaard K, Rasmussen J (2012) Nitrogen transfer from forage legumes to nine neighbouring plants in a multi-species grassland. Plant Soil 350: 71-84.

Proulx RA, Naeve SL (2009) Pod removal, shade, and defoliation effects on soybean yield, protein, and oil. Agron J 101: 971-978.

Rao MA, Scelza R, Gianfreda L (2014) Soil Enzymes. Enzymes in Agricultural Sciences. OMICS Group eBooks, Foster City, pp. 10–43.

R Core Team. 2023. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Richards JH (1993) Physiology of plants recovering from defoliation. Proc. XVII international grassland congress. Palmerston North, N.Z. p. 85-94.

Rimi F, Macolino S, Leinauer B, Lauriault LM, Ziliotto U (2014) Fall Dormancy and Harvest Stage Impact on Alfalfa Persistence in a Subtropical Climate. Agron J 106: 1258-1266.

Rochon JJ, Doyle CJ, Greef JM, Hopkins A, Molle G, Sitzia M, Scholefield D, Smith CJ (2004) Grazing legumes in Europe: a review of their status, management, benefits, research needs and future prospects. Grass Forage Sci 59: 197-214.

Rognes T, Flouri T, Nichols B, Quince C, Mahé F (2016) VSEARCH: a versatile open source tool for metagenomics. PeerJ 4: e2584.

Sanderson MA, Brink G, Stout R, Ruth L (2013) Grass–legume proportions in forage seed mixtures and effects on herbage yield and weed abundance. Agron J 105:1289-1297.

Saiya-Cork KR, Sinsabaugh RL, Zak DR (2002) The effects of long term nitrogen deposition on extracellular enzyme activity in an Acer saccharum forest soil. Soil Biol Biochem 34: 1309-1315.

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, et al. (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75: 7537-7541.

Serraj R (2003) Effects of drought stress on legume symbiotic nitrogen fixation: physiological mechanisms. Indian J Exp Biol 41: 1136-1141

Shi S, Nuccio E, Herman DJ, Rijkers R, Estera K et al. (2015) Successional trajectories of rhizosphere bacterial communities over consecutive seasons. mBio 6: e00746.

Sleugh B, Moore KJ, George JR, Brummer EC (2000) Binary legume–grass mixtures improve forage yield, Quality, and seasonal distribution. Agron. J 92: 24-29.

Stagnari F, Maggio A, Galieni A, Pisante M (2017) Multiple benefits of legumes for agriculture sustainability: an overview. Chem. Biol Technol Agric 4: 2.

Sulieman S, Tran LSP (2016). Legume nitrogen fixation in a changing environment: achievements and challenges. doi:10.1007/978-3-319-06212-9.

Teixeira EI, Moot DJ, Brown HW (2007) Defoliation frequency and season affected radiation use efficiency and dry matter partitioning to roots of lucerne (*Medicago sativa* L.) crops. Europ J Agron 28: 103-111.

Thilakarathna MS, McElroy MS, Chapagain T, Papadopoulos YA, Raizada MN (2016) Belowground nitrogen transfer from legumes to non-legumes under managed herbaceous cropping systems. a review. Agron Sustain Dev 36: 58.

Thilakarathna MS, Moroz N, Raizada MN (2017) A biosensor-based leaf punch assay for glutamine correlates to symbiotic nitrogen fixation measurements in legumes to permit rapid screening of rhizobia inoculants under control conditions. Front Plant Sci 08: 1714.

Thilakarathna MS, Raizada MN (2018) Visualizing glutamine accumulation in root systems involved in the legume–Rhizobia symbiosis by placement on agar embedded with companion biosensor cells. Phytobiomes J 2: 117-128.

Thilakarathna MS, Raizada MN (2019) A biosensor-based assay (GlnLux-Agar) shows defoliation triggers rapid release of glutamine from nodules and young roots of forage legumes. Phytobiomes J 3: 85-91.

Tian K, Kong X, Yuan L, Lin H, He Z, Yao B, Ji Y, Yang J, Sun S, Tian X (2019) Priming effect of litter mineralization: the role of root exudate depends on its interactions with litter quality and soil condition. Plant Soil 440: 457-471.

Vance CP, Heichel GH, Barnes DK, Bryan JW, Johnson LE (1979) Nitrogen fixation, nodule development, and vegetative regrowth of alfalfa (*Medicago sativa* L.) following harvest. Plant Physiol 64: 1-8.

Ward NL, Challacombe JF, Janssen PH, Henrissat B, Coutinho PM, Wu M, Xie G, Haft DH, Sait M, Badger J, et al. (2009) Three genomes from the phylum Acidobacteria provide insight into the lifestyles of these microorganisms in soils. Appl Environ Microbiol 75: 2046-2056.

Weiss S, Xu ZZ, Peddada S, Amir A, Bittinger K, Gonzalez A, Lozupone C, Zaneveld JR, Vázquez-Baeza Y, et al. (2017) Normalization and microbial differential abundance strategies depend upon data characteristics. Microbiome 5:27.

Wilson CH, Strickland MS, Hutchings JA, Bianchi TS, Flory SL (2018) Grazing enhances belowground carbon allocation, microbial biomass, and soil carbon in a subtropical grassland. Glob. Change Biol. 24: 2997-3009.

Xu X, Sherry RA, Niu S, Li D, Luo Y (2013) Net primary productivity and rain-use efficiency as affected by warming, altered precipitation, and clipping in a mixed-grass prairie. Global Change Biol 19: 2753-2764.

Xu F, Guo W, Wang R, Xu W, Du N, Wang Y (2009) Leaf movement and photosynthetic plasticity of black locust (*Robinia pseudoacacia*) alleviate stress under different light and water conditions. Acta Physiol Plant 31:553-563

Xun W, Zhao J, Xue C, Zhang G, Ran W, Wang B, Shen Q, Zhang R (2016) Significant alteration of soil bacterial communities and organic carbon decomposition by different long-term fertilization management condition of extremely low productivity arable soil in South China. Envi Micro 18: 1907-1917

Zhang Y, Lu W, Zhang H, Zhou J, Shen Y (2018) Grassland management practices in Chinese steppes impact productivity, diversity and the relationship. Front Agron Sci Eng 5: 57-63.

Chapter 4 – General discussion

Forage legumes have been crucial players in achieving sustainable agriculture because of their ability to fix, store, and transfer biological N into the soil and adjacent plants (Rochon et al., 2004; Rubiales and Mikic, 2015). In addition to the ecological services they provide, they are proficient in protein production for animal consumption (Tharanathan and Mahadevamma, 2003). The effective use of forage legumes in traditional farming, however, is hindered by abiotic and biotic stress factors (Zahran, 1999; Laranjo et al., 2014; Kasper et al., 2019; Thilakarathna et al., 2016; Lesk et al., 2016), which can limit the benefits growers and consumers can gain.

Drought has been known as the most critical abiotic threat to food security globally by constraining crop yield (Micheletto et al., 2007; Farooq et al., 2016; Ortiz-Bobea et al., 2021). Plants, in general, rely on water to perform different biochemical and physiological processes, and soil moisture limitation can influence these variables, resulting in reduced forage yield and quality (Enebe and Babalola, 2018; Farooq et al., 2009; Jaleel et al., 2009). On the other hand, defoliation stress or crown damage is a biotic stress factor that forage plants will inevitably experience, from pest damage to anthropogenic-led activities like livestock overgrazing practices (Thilakarathna et al., 2016). Defoliation can strongly influence the balance of resource allocation between the above- and below-ground tissues, which can alter growth and yield (Gordon et al., 1990; Richards, 1993; King and Purcell, 2001; Aranjuelo et al., 2015). Moreover, the plant response to these stress factors can have a consequential influence on the below-ground plant-microbe interaction dynamics, as plants are closely linked to the soil microbes (Ma et al., 2018; Berg et al., 2016). Therefore, in this study, we separately assessed the nodulation, growth, and plant physiological responses at the flowering stage to drought and defoliation stress and the alteration of soil microbiome community structure and extracellular enzyme activities. Data from this study can be crucial in developing a better sustainable management strategy involving crop rotations, grazing management, and fertilizer management.

Effective use of forage legumes for biological N fixation is tied to the success of the nodulation process. Drought and defoliation have been known to affect nodulation in legumes negatively (Lumactud et al., 2023; Fernández-Luqueño et al., 2008; Marquez-Garcia et al., 2015; Aranjuelo et al., 2015; Chesney and Nygren, 2002). This study has documented a significant decline in alfalfa and red clover nodule numbers following drought and defoliation stress. A

healthy rhizobial soil population is a prerequisite to initiating nodulation (Rehman and Nautiyal, 2002); extended period of drought can reduce the survival of rhizobial bacteria, thus effectively reducing nodulation (Herrmann et al., 2014; Kasper et al., 2019). We also observed that drought did not affect alfalfa nodule weight, contrasting with the reduction effect on red clover nodules. In light of the nodule number decline, maintaining nodule weight is perhaps a form of adaptive response in alfalfa sto sustain the N fixation rate (Lumactud et al., 2023). On the other hand, defoliation influences both nodule initiation and already established nodules. The loss of photosynthetic tissues results in a decline in resource allocation to the roots, inducing nodule senescence and, consequently, the reduction in nodule number and dry weight (Chesney and Nygren, 2002). In addition to the decline in nodulation, the reduced resource allocation also results in reduced nitrogenase activity due to the disruption of O_2 permeability (Denison et al., 1992; Nygren et al., 2000; Sulieman and Tran, 2016) and the intensifying of tissue senescence because of the increase in protease and nitrate reductase (Vance et al., 1979).

Defoliation stress has an apparent adverse effect on plant physiological processes because of the loss of photosynthetic tissues (Gordon et al., 1990; Richards, 1993). However, during drought, plants have different drought tolerance mechanisms that can be influenced by moisture limitation. In this study, we considered four different physiological parameters related to drought tolerance: linear electron flow (LEF) as a proxy for photosynthesis, leaf chlorophyll content, quantum yield (ΦII), and non-photochemical quenching (ΦNPQ). Drought is generally considered to have a deleterious effect on LEF and photosynthesis rate by limiting carbon influx through the stomata and by reducing the amount of leaf chlorophyll (Zargar et al., 2017; Zhang C et al., 2018; Wang et al., 2018; Jacques et al., 2022). The results of our study did not agree with these findings as we reported that LEF was affected by either moderate or severe drought intensities. One possible explanation for these contradictory findings is that our drought intensities were insufficient to induce a significant adverse effect on LEF and that alfalfa and red clover plants acclimated along the three-week drought treatment. A parameter that has a link to photosynthesis and could explain the non-significant drought effect on it is the chlorophyll content. Interestingly, alfalfa leaf chlorophyll content has positively responded to moderate drought and was significantly improved, consistent with previous literature (Vadez et al., 2000; King and Purcell, 2005; Sulieman and Tran, 2016). The increase in chlorophyll can lead to feedback inhibition of N fixation because of the accumulation of N in the leaves. These N forms function as chemical signal molecules inciting the

inhibition of SNF under drought (Neo and Layzell, 1997; Vadez et al., 2000; Serraj et al., 2001; King and Purcell, 2005; Gil-Quintana et al., 2013). Furthermore, ΦII and ΦNPQ are tied to photosynthesis efficiency. These efficiency variables have an inverse relationship, and we report that a higher ΦNPQ results in a lower ΦII, consistent with a study on cowpeas (Mwale et al., 2017).

Drought is known to influence plant biomass negatively. We observed a steep decrease in shoot biomass of about 59% and 34% under severe drought in alfalfa and red clover, respectively. A similar shift was found under severe drought stress in root biomass, which was reduced by 40% and 30% compared to well-watered plants. These results corroborate previous studies (Mouradi et al., 2016; Begum et al., 2019). The reducing effect of drought on plant biomass is linked to its influence on plant cell metabolism, which is tied to cell growth (Vaseva et al., 2011; Ashraf and O'Leary, 1996). As previously mentioned, defoliation alters the balance of the source-sink relation of the above- and below-ground tissues. In livestock agriculture, the total shoot biomass consumed is an important criterion in choosing forage species. Our study reports a significant increase in final shoot biomass following mild defoliation intensity. This suggests that mild defoliation promotes shoot growth because recovery from crown damage demands that most of the nutrient reserves and production be reallocated toward shoot tissues to support regrowth (Ourry et al., 1994; Meuriot et al., 2005; Teixeira et al., 2007). This suggests an accumulation of N in the shoot tissues, which our study confirms by the increased shoot N content and non-significant change in N fixation.

On the contrary, drought reduces SNF rates in legumes, resulting in a decline in shoot N content. Several factors can be at play in inhibiting N_2 fixation under drought and even following defoliation should SNF be observed to decline. Drought and defoliation contribute to the decline in nodule sucrose synthase, resulting in the shortage of carbon substrates needed to facilitate SNF (González et al. 2015; Kunert et al. 2016). Another contributor is oxygen limitation. As previously stated, the nodule requires a microaerobic environment to maintain the activity of the nitrogenase enzyme, which deactivates in the presence of oxygen, and to sustain the respiration of the bacteroid (Sulieman and Tran, 2016). Drought and defoliation are found to induce senescence of nodule tissue by inducing the production and accumulation of the ABA hormone responsible for cell death (Serraj, 2003; Gómez-Cadenas et al., 2000; Iglesias et al., 2003; Ren et al., 2007), leading to the loss of cell turgor pressure and causing the increase of oxygen diffusion (Pankhurst and Sprent, 1975; Serraj et al., 1999; Serraj, 2003).

Improving water use efficiency (WUE) is one of the critical drought tolerance mechanisms adopted by plants. Carbon isotope discrimination (CID) determination is an accepted method for evaluating WUE in C_3 plants and is inversely proportionate to one another (Hubick et al., 1986; Moghaddam et al., 2013). A lower CID indicates improved WUE during drought stress (Kaler et al., 2018). Our study agrees with this as we report a lower CID under water deficit, suggesting a higher WUE in both plants under drought than well-watered plants.

Soil extracellular enzyme activity (EEA) is a strong predictor for soil health, providing the leading force for nutrient cycling, including C, N, and P (Burns et al. 2013). The effects of drought on EEA varies from research to research. Some studies report a sharp decrease in oxidoreductase activity while there is an increase in hydrolase activities under soil drought. On the other hand, Geisseler et al. (2011) concluded that drought greatly increases soil EEAs. The present study observed that drought did not affect soil EEA except for a significant decrease in activity of NAG under both 40% and 20% FC treatment in alfalfa and a significant reduction of C-acquisition hydrolase (Cello) under 20% FC in red clover pots. On the other hand, the activity of C and P cycling enzymes was increased following defoliation, which corroborates with field studies on defoliation (Hewins et al. 2016). The increase in cellobiosidase and glucosidase could be attributed to the increase in C sources due root exudation and root tissue decomposition induced by defoliation (Belsky 1986). Like the response of C enzymes, the increase in phosphatase activity can be linked to the increased organic P availability from exudates and plant tissue. It was shown that P uptake increases following defoliation (Li et al., 2010). The probable increase in plant and bacteria P demand may have led to increased bacterial activity in P cycling (Hewins et al., 2016). The defoliation PCA analysis showed that EEAs were positively correlated with soil available N, suggesting an increase in root exudation following defoliation that changes microbial activity in the soil (Kuzyakov and Xu, 2013). Finally, NAG was observed to have not been affected by the defoliation treatments. This non-significant effect can be tied to the lack of Acidobacteria species specialized in N cycling (Kang et al., 2021; Ward et al., 2009; Belova et al., 2018) or due to the rapid mineralization of N (Bai et al., 2021; Kang et al., 2021; Hewins et al., 2016). In addition, defoliation may have triggered a rapid release of fixed nitrogen through nodules and roots tissues (Thilakarathna et al., 2019), causing an increase in available nitrogen in the soil that plants and microbes will use.

Soil is a hub for microorganisms, both harmful and beneficial (Bar-On, 2018; Wang et al., 2020; Trasar-Cepeda et al., 2008; Kabiri et al., 2016). Plants can selectively recruit those with growth-promoting impacts through rhizodeposition of exudates in the rhizosphere (Edwards et al., 2015; Finkel et al., 2017). These growth-promoting activities can be through enzyme activities induced by the release of substrates by microorganisms (Kwiatkowski et al., 2020; Mndzebele et al., 2020; Harasim et al., 2020). The present study has found that the increased rhizodeposition of exudates into the soil due to the senescence of tissues, increasing the pool of N compounds in the soil (Wichern et al., 2008; Fustec et al., 2010; Ayres et al., 2004, 2007; Carrillo et al., 2011). Accompanying these N compounds are other substances and molecules that plants can use to exert pressure in microorganism recruitment (Hamilton and Frank, 2001; Dennis et al., 2010; Huang et al., 2014; Shi et al., 2015). The noticeable distinction between the bacterial diversity of the rhizosphere and the bulk soil suggests that under stress, alfalfa and red clover selectively recruited specific bacterial species into their root zone (Finkel et al., 2017). The present study found a contrasting results on the abundance of Acidobacteria. Acidobacteria are susceptible to drought, reducing their abundance (Acosta-Martínez et al., 2014; Barnard et al., 2013; Maestre et al., 2015), which agrees with our findings. The decrease in abundance can be linked to the reduced N-acetylglucosaminadase (NAG) activity involved in N cycling in the soil because it is revealed that Acidobacteria are prominent players in N cycling (Kang et al., 2021; Ward et al., 2009; Belova et al., 2018). In defoliation, on the one hand, the abundance of Acidobacteria species has increased due to their oligotrophic nature favoring the low labile C availability caused by the allocation of photosynthates to the above-ground tissue, as opposed to below-ground or the loss of C resources due to deposition and root senescense (Aranjuelo et al., 2015; Xu et al. 2013; Fierer et al., 2007; Xun et al., 2016).

Aside from Acidobacteria, enrichment of *Nocardioidaceae* from the phylum Actinobacteria in the drought-treated red clover rhizosphere is consistent with previous studies (Bouskill et al., 2016; Kavamura et al., 2013; Santos-Medellín et al., 2021; Xu et al., 2018). Their enrichment is likely tied to their morphology and physiology. Actinobacteria generally have thick peptidoglycan cell walls, being Gram-negative bacteria, and because their capacity to accumulate osmolytes allows them to be highly resistant to desiccation by cell dehydration (Hartman and Tringe, 2019). Red clover may have recruited them because of their capacity to accumulate various

metabolites through their siderophores in hostile environments, which can prove to be beneficial for drought tolerance in plants (Lewin et al., 2016).

A greenhouse pot experiment such as this study poses some limitations that could elicit different results. Plants try to be resilient under drought by rooting deeper into the soil for water sources; thus, the size of the pot could exacerbate the deleterious effect of soil drought, which we have observed. A controlled environment could also hinder other changes that drought and defoliation could induce and since forages are typically grown on farms, plants are exposed to various uncontrolled variables. Another limitation of the study's design is the parameter limitations that the default MultispeQ can analyze. As mentioned, plants use diverse drought tolerant mechanisms, and exploring other parameters, such as actual photosynthesis rate, stomatal closure, and gas exchange, can provide more evidence on the effects of drought on forage legumes. There is also a lack of a method for studying resource allocation between above- and below-ground tissues and observing rhizodeposition rate. Lastly, we limited our microbial study to bacteria. The soil is home to an amalgamation of microorganism species, such as fungal and archaea species, that are also known to be strongly linked with the root system of plants and are beneficial for alleviating the effects of stress factors.

As a future direction, we can take a similar approach and observable parameters but use a more diverse forage legume variety panel. For drought studies, perhaps more varying drought intensities, while defoliation is a combination of intensity and frequency of trimmings. Furthermore, it would be more impactful to conduct field trials; that way, we can emulate how these varieties/species could behave in an actual field scenario under these stress factors. In line with this, forage legumes are usually used as mixed crops with grasses; we could design a study aiming to observe how these stress factors influence both beneficial and competitive relationships between forage legumes and grasses and their influence on the plant-soil dynamics. As suggested by our study, alfalfa and red clover potentially have recruited specific bacteria with growth-promoting impact. Therefore, it will be a great future endeavor to identify the specific chemical compounds these species release and use to attract these microbes.

In summary, the present study provides evidence that drought and defoliation elicit negative and positive alterations in plant responses at the flowering stage under stress. Both stress factors negatively impacted nodulation in alfalfa and red clover. Drought has an overall adverse effect on both shoot and root biomass, while defoliation only reduces root biomass while

improving the final total biomass harvested. Furthermore, drought is found to be deleterious to N-acetyl-glucosaminidase in alfalfa soil and β-D cellobiosidase activity in red clover, which can be linked to the decrease in Acidobacteria species abundance under soil moisture deficit. On the other hand, defoliation has an enhancing effect on carbon cycling enzymes: β-1, 4-glucosidase, β-D cellobiosidase, and phosphate cycling enzyme phosphatase. Collectively, these findings enhance our understanding of the interaction between above- and below-ground parameters and will provide insights that can be valuable in management protocols.

References

Acosta-Martínez V, Cotton J, Gardner T, Moore-Kucera J, Zak J, Wester D, Cox S (2014) Predominant bacterial and fungal assemblages in agricultural soils during a record drought/heat wave and linkages to enzyme activities of biogeochemical cycling. Appl Soil Ecol 84:69–82. https://doi.org/10.1016/j.apsoil.2014.06.005

Adentuji AT, Ncube B, Mulidzi R, Lewu FB (2020) Potential use of soil enzymes as soil quality indicators in agriculture. Front Soil Environ Micro 6: 58-62.

Ankati S, Podile AR (2019) Metabolites in the root exudates of groundnut change during interaction. J Plant Physiol 243: 153057.

Alkorta I, Aizpurua A, Riga P, Albizu I, Amézaga I, Garbisu C (2003) Soil enzyme activities as biological indicators of soil Health. Rev. Environ. Health18: 65–73

Aranjuelo I, Molero G, Erice G, Aldasoro J, Arrese-Igor C, Nogués S (2015) Effect of shoot removal on remobilization of carbon and nitrogen during regrowth of nitrogen-fixing alfalfa. Physiology Plant 153:91-104.

Arrese-Igor C, González EM, Gordon AJ, Minchin FR, Gálvez L, Royuela M, Cabrerizo PM, Aparicio-Tejo PM (1999) Sucrose synthase and nodule nitrogen fixation under drought and other environmental stresses. Symbiosis 27: 189–212

Ashraf M, O'Leary JW (1996) Effect of drought stress on growth, water relations, and gas exchange of two lines of sunflower differing in degree of salt tolerance. Int J Plant Sci 157:729–732. https://doi.org/10.1086/297395

Ayres E, Heath J, Possell M, Black HIJ, Kerstiens G, Bardgett RD (2004) Tree physiological responses to above-ground herbivory directly modify below-ground processes of soil carbon and nitrogen cycling. Ecol Lett 7: 469-479.

Ayres E, Dromph KM, Cook R, Ostle N, Bardgett RD (2007) The influence of below-ground herbivory and defoliation of a legume on nitrogen transfer to neighbouring plants. Funct Ecol 21: 256-263.

Bachar A, Al-Ashhab A, Soares MIM, Sklarz MY, Angel R, Ungar ED, Gillor O (2010) Soil microbial abundance and diversity along a low precipitation gradient. Microb Ecol 60:453–461. https://doi.org/10.1007/S00248-010-9727-1

Badri DV, Vivanco JM (2009) Regulation and function of root exudates. Plant Cell Envi 32: 666–681.

Bai X, Dippold MA, An S, Wang B, Zhang H, Loeppmann S (2021) Extracellular enzyme activity and stoichiometry: the effect of soil microbial element limitation during leaf litter decomposition. Ecol Indic 121: 107200.

Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. Annual Review of Plant Biology. 57: 233–266.

Bar-On YM, Philips R, Milo R (2018) The biomass distribution on Earth. PNAS 115: 6505-6511.

Barnard RL, Osborne CA, Firestone MK (2013) Responses of soil bacterial and fungal communities to extreme desiccation and rewetting. ISME J 7:2229–2241. https://doi.org/10.1038/ISMEJ.2013.104

Basak R, Wahid KA, Dinh A, Soolanayakanahally R, Fotouhi R, Mehr AS (2020) Rapid and efficient determination of relative water contents of crop leaves using electrical impedance spectroscopy in vegetative growth stage. Remote Sens 12:1753. https://doi.org/10.3390/rs12111753

Bausenwein U, Gattinger A, Langer U, Embacher A, Hartmann H-P, Sommer M, Munch JC, Schloter M (2008) Exploring soil microbial communities and soil organic matter variability and interactions in arable soils under minimum tillage practice. Appl Soil Ecol 40: 67-77.

Baysdorfer C, Bassham JA (1985) Photosynthate supply and utilization in alfalfa: a developmental shift from a source to a sink limitation of photosynthesis. Plant Physiol 77: 313-317.

Begum N, Qin C, Ahanger MA, Raza S, Khan MI, Ashraf M, Ahmed N, Zhang L (2019) Role of arbuscular mycorrhizal fungi in plant growth regulation: implications in abiotic stress tolerance. Front Plant Sci 10:1068. https://doi.org/10.3389/fpls.2019.01068

Beier C, Beierkuhnlein C, Wohlgemuth T, Penuelas J, Emmett B, Körner C, de Boeck H, Christensen JH, Leuzinger S, Janssens IA et al (2012) Precipitation manipulation experiments–challenges and recommendations for the future. Ecol Lett 15:899–911. https://doi.org/10.1111/j.1461-0248.2012.01793.x

Belova SE, Ravin NV, Pankratov TA, Rakitin AL, Ivanova AA, Beletsky AV, Mardanov AV, Sinninghe Damsté JS, Dedysh SN (2018) Hydrolytic capabilities as a key to

environmental success: chitinolytic and cellulolytic *Acidobacteria* from acidic sub-arctic soils and boreal peatlands. Front Microbiol 19: 2775.

Belsky AJ (1986) Does Herbivory Benefit Plants? A review of the evidence. Amer Soc Nat 12: 870-892.

Berg G, Grube M, Schloter M, Smalla K (2014) Unraveling the plant microbiome: looking back and future perspectives. Front Microbiol 5:148. https://doi.org/10.3389/fmicb.2014.00148

Berg G, Rybakova D, Grube M, Köberl M (2016) The plant microbiome explored: implications for experimental botany. J Exp Bot 67:995–1002. https://doi.org/10.1093/jxb/erv466

Bertin C, Yang X, Weston, LA (2003) The role of root exudates and allelochemicals in the rhizosphere. Plant Soil 256: 67–83.

Black AS, Laidlaw AS, Moot DJ, O'Kiely P (2009) Comparative growth and management of white and red clovers. IJAFR 48: 149-166.

Bogati K, Walczak M (2022) The impact of drought stress on soil microbial community, enzyme activities and plants. Agron 12:1–26. https://doi.org/10.3390/agronomy12010189

Bouskill NJ, Wood TE, Baran R, Ye Z, Bowen BP, Lim HC, Zhou J, Van Nostrand JD, Nico P, Northen TR et al (2016) Belowground response to drought in a tropical forest soil. I. changes in microbial functional potential and metabolism. Front Microbiol 7:525. https://doi.org/10.3389/fmicb.2016.00525

Bowles TM, Acosta-Martínez V, Calderón F, Jackson LE (2014) Soil enzyme activities, microbial communities, and carbon and nitrogen availability in organic agroecosystem across an intensively-managed agricultural landscape. Soil Biol Biochem 68: 252:262.

Britto DT, Kronzucker HJ (2002) NH4+ toxicity in higher plants: a critical review. J Plant Phys 159: 567-584

Brophy LS, Heichel GH (1989) Nitrogen release from roots of alfalfa and soybean grown in sand culture. Plant Soil 116: 77–84

Burns RG (1982) Enzyme activity in soil: location and a possible role in microbial ecology. Soil Biol Biochem 14:423–427. https://doi.org/10.1016/0038-0717(82)90099-2

Burns RG, DeForest JL, Marxsen J, Sinsabaugh RL, Stromberger ME, Wallenstein MD, Weintraub MN, Zoppini A (2013) Soil enzymes in a changing environment: current knowledge and future directions. Soil Biol Biochem 58:216–234. https://doi.org/10.1016/j.soilbio.2012.11.009

Camargo JA, Alonso A (2006) Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: a global assessment. Envi Int 32: 831-849

Cameron KC, Di HJ, Moir JL (2013) Nitrogen losses from the soil/plant system: a review. Ann Appl Biol 162:145–173. https://doi.org/10.1111/aab.12014

Canarini A, Kaiser C, Merchant A, Richter A, Wanek W (2019) Root exudation of primary metabolites: mechanisms and their roles in plant responses to environmental stimuli. Front Plant Sci 10: 157.

Carrillo Y, Jordan CF, Jacobsen KL, Mitchell KG, Raber P (2011) Shoot pruning of a hedgerow perennial legume alters the availability and temporal dynamics of root-derived nitrogen in a subtropical setting. Plant Soil 345: 59-68.

Cavaglieri L, Orlando J, Etcheverry M (2009) Rhizosphere microbial community structure at different maize plant growth stages and root locations. Microbiol Res 164:391–399. https://doi.org/10.1016/j.micres.2007.03.006

Cesco S, Neumann G, Tomasi N, Pinton R, Weisskopf L (2010) Release of plant-borne flavonoids into the rhizosphere and their role in plant nutrition. Plant Soil 329: 1-25.

Chalk PM (1998) Dynamics of biologically fixed N in legume-cereal rotations: a review. Aust J of Agri Res 49: 303-316.

Chaparro JM, Badri DV, Bakker MG, Sugiyama A, Manter DK, Vivanco JM (2013) Root exudation of phytochemicals in Arabidopsis follows specific patterns that are developmentally programmed and correlate with Soil Microbial functions. PLoS ONE 8:e55731. https://doi.org/10.1371/journal.pone.0055731

Chaparro JM, Badri DV, Vivanco JM (2014) Rhizosphere microbiome assemblage is affected by plant development. ISME J 8:790–803. https://doi.org/10.1038/ismej.2013.196

Charpentier M, Oldroyd GE (2013) Nuclear calcium signaling in plants. Plant Physiol 163:496-503.

Chesney P, Nygren P (2002) Fine root and nodule dynamics of *Erythrina poeppigiana* in an alley cropping system in Costa Rica. Agrifor Syst 56: 259-269.

Chong J, Liu P, Zhou G, Xia J (2020) Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. Nat Protoc 15:799–821. https://doi.org/10.1038/s41596-019-0264-1

Christie BR, Bennett RJ (1984) OAC Minto Alfafla. Can J Plant Sci 64:419–421

Chroma L, Mackova M, Kucerova P (2002) In Der Wiesche, C.; Burkhard, J.; Macek, T. Enzymes in plant metabolism of PCBs and PAHs. Acta Biotechnol 22: 35–41

Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, Brown CT, Porras-Alfaro A, Kuske CR, Tiedje JM (2014) Ribosomal database project: data and tools for high throughput rRNA analysis. Nucleic Acid Res 42:D633–D642. https://doi.org/10.1093/nar/gkt1244

Condron L, Stark C, O'Callaghan M, Clinton P, Huang Z (2010) The Role of Microbial Communities in the Formation and Decomposition of Soil Organic Matter. In: Dixon, G., Tilston, E. (eds) Soil Microbiology and Sustainable Crop Production. Springer, Dordrecht. p.81

Corel E, Lopez P, Méheust R, Bapteste E (2016) Network-thinking: graphs to analyze microbial complexity and evolution. Trends in Micro 24: 224-237.

Courty PE, Smith P, Koegel S, Redecker D, Wipf D (2014) Inorganic nitrogen uptake and transport in beneficial plant root-microbe interactions. Crit Rev Plant Sci 34: 4-16.

Cralle HT, Heichel GH (1981) Nitrogen fixation and vegetative regrowth of alfalfa and birdsfoot trefoil after successive harvests or floral debudding. Plant Physiol 67: 898-905.

Davidson JL, Milthorpe FL (1996) The effect of defoliation on the carbon balance in *Dactylis glomerata.* Annals Bot 8: 185-198.

Dénarié J, Debellé F, Promé JC (1996) Rhizobium lipo-chitooligosaccharide nodulation factors: signaling molecules mediating recognition and morphogenesis. Annu Rev Biochem 65:503-35.

de Freitas IC, Ferreira EA, Alves MA, de Oliveira JC, Frazão LA (2023) Growth, nodulation, production, and physiology of leguminous plants in integrated production systems. Agrosyst Geosci Environ 6:e20343. https://doi.org/10.1002/agg2.20343

De Haan RL, Schuiteman MA, Vos RJ (2017) Residual soil nitrate content and profitability of five cropping systems in northwest Iowa. PLoS ONE 12:e0171994. https://doi.org/10.1371/journal.pone.0171994

De Vries FT, Wallenstein MD (2017) Below-ground connections underlying above-ground food production: a framework for optimising ecological connections in the rhizosphere. J Ecol 105:913–920. https://doi.org/10.1111/1365-2745.12783

De Vries FT, Griffiths RI, Knight CG, Nicolitch O, Williams A (2020) Harnessing rhizosphere microbiomes for drought-resilient crop production. Science 368:270–274

del Castillo LD, Hunt S, Layzell DB (1994) The role of oxygen in the regulation of nitrogenase activity in drought-stressed soybean nodules. Plant Physiol 106:949–955

Denison RF, Hunt S, Layzell DB (1992) Nitrogenase activity, nodule respiration, and O_2 permeability following detopping of alfalfa and birdsfoot trefoil. Plant Physiol 98: 894-900.

Dennis PG, Miller AJ, Hirsch PR (2010) Are root exudates more important than other sources of rhizodeposits in structuring rhizosphere bacterial communities? FEMS Microbiol Ecol 72:313–327. https://doi.org/10.1111/j.1574-6941.2010.00860.x

Dey R, Lewis SC, Arblaster JM, Abram NJ (2019) A review of past and projected changes in Australia's rainfall. Wiley Interdiscip Rev Clim Change 10:e00577. https://doi.org/10.1002/wcc.577

Dias PC (1996) Sources and sinks in population biology. Trends Ecol Evol 11: 326–330

Dick RP (1994) Soil enzyme activities as indicators of soil quality. Defin Soil Qual A Sustain Environ 35: 107–124

Dick RP (1997) Soil Enzyme Activities as Integrative Indicators of Soil Health. In Biological Indicators of Soil Health; Pankhurst, C.E., Doube, B.M., Gupta, V.V.S.R., Eds.; USA7 CAB Internat: Wallingford, UK; pp. 121–156.

Doran JW, Zeiss MR (2000) Soil health and sustainability: managing the biotic component of soil quality. Appl Soil Ecol 15: 3-11

Downie JA (2005) Legume haemoglobins: symbiotic nitrogen fixation needs bloody nodules. Curr Biol 15: 196-198.

Dubach M, Russelle MP (1994) Forage legume roots and nodules and their role in nitrogen transfer, Agron. J. 86, 259–266.

Edwards J, Johnson C, Santos-Medellin C, Sundaresan V (2015) Structure, variation, and assembly of the root-associated microbiomes of rice. PNAS 112: E911-E920

Egamberdieva D, Renella G, Wirth S, Islam R (2010) Enzyme activities in the rhizosphere of plants. In Soil Enzymology; Shukla, G., Varma, A., Eds.; Soil Biology; Springer Berlin Heidelberg: Berlin, Heidelberg, 2010; Volume 22, pp. 149–166.

Emerich DW, Krishnan H, Peoples MB, Hauggaard-Nielsen H, Jensen ES (2009) The potential environmental benefits and risks derived from legumes in rotations. Agron Monograph 52: 349-385

Enebe MC, Babalola OO (2018) The influence of plant growth-promoting rhizobacteria in plant tolerance to abiotic stress: a survival strategy. Appl Microbiol Biotech 102:7821–7835. https://doi.org/10.1007/s00253-018-9214-z

Fageria NK (2014) Nitrogen harvest index and its association with crop yields. J Plant Nutr 37:795–810. https://doi.org/10.1080/01904167.2014.881855

Farooq M, Gogoi N, Barthakur S, Baroowa B, Bharadwaj N, Alghamdi SS, Siddique KHM (2016) Drought stress in grain legumes during reproduction and grain filling. J Agron Crop Sci 203: 81-102.

Farooq M, Gogoi N, Barthakur S, Baroowa B, Bharadwaj N, Alghamdi SS, Siddique KHM (2017) Drought stress in grain legumes during reproduction and grain filling. J Agro Crop Sci 203:81–102. https://doi.org/10.1111/jac.12169

Farooq M, Wahid A, Kobayashi N, Fujita D, Basra SMA (2009) Plant drought stress: effects, mechanisms and management. Agron Sustain Dev 29:185–212. https://doi.org/10.1051/agro:2008021

Fernández-Luqueño F, Dendooven L, Munive A, Corlay-Chee L, Serrano-Covarrubias LM, Espinosa-Victoria D (2008) Micro-morphology of common bean (*Phaseolus vulgaris* L.) nodules undergoing senescence. Acta Physiol Plant 30:545–552. https://doi.org/10.1007/s11738-008-0153-7

Fierer N, Bradford MA, Jackson RB (2007) Toward an ecological classification of soil bacteria. Ecol 88: 1354-1364.

Fierer N, Schimel JP, Holden PA (2003) Variations in microbial community composition through two soil depth profiles. Soil Biol Biochem 35:167–176. https://doi.org/10.1016/s0038-0717(02)00251-1

Finkel OM, Castrillo G, Paredes SH, González IS, Dangl JL (2017) Understanding and exploiting plant beneficial microbes. Curr Opin Plant Biol 38:155–163. https://doi.org/10.1016/j.pbi.2017.04.018

Fitzpatrick CR, Copeland J, Wang PW, Guttman DS, Kotanen PM, Johnson MTJ (2018) Assembly and ecological function of the root microbiome across angiosperm plant species. PNAS 115:E1157–E1165. https://doi.org/10.1073/pnas.1717617115

Follett RF, Delgado JA (2002) Nitrogen fate and transport in agricultural systems. J Soil Water Conserv 57: 402–408

Fustec J, Lesuffleur F, Mahieu S, Cliquet JB (2010) Nitrogen rhizodeposition of legumes. A review. Agron Sustain Dev 30:57–66

Gage DJ (2004) Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. Micro Mol Biol Rev 68: 280-300.

Gaiero JR, McCall CA, Thompson KA, Day NJ, Best AS, Dunfield KE (2013) Inside the root microbiome: bacterial root endophytes and plant growth promotion. Am J Bot 100:1738–1750. https://doi.org/10.3732/ajb.1200572

Gattinger A, Palojärvi A, Schloter M (2008) Soil microbial communities and related functions. Perspectives for Agroecosystem Management. Amsterdam, Netherlands: Elsevier, pp. 279–292

Geisseler D, Horwath WR, Scow KM (2011) Soil moisture and plant residue addition interact in their effect on extracellular enzyme activity. Pedobiologia (Jena) 54:71–78. https://doi.org/10.1016/j.pedobi.2010.10.001

German DP, Weintraub MN, Grandy AS, Lauber CL, Rinkes ZL, Allison SD (2011) Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. Soil Biol Biochem 43:1387–1397. https://doi.org/10.1016/j.soilbio.2011.03.017

Gianfreda L (2015) Enzymes of Importance to Rhizosphere Processes. J Soil Sci Plant Nutr 15: 283–306

Gil-Quintana E, Larrainzar E, Seminario A, Díaz-Leal JL, Alamillo JM, Pineda M, et al. (2013) Local inhibition of nitrogen fixation and nodule metabolism in drought-stressed soybean. J Exp Bot 64: 2171–2182

Giller KE (2001) Nitrogen fixation in tropical cropping systems. CAB International, Wallingford, UK. pp. 423

Gogoi N, Baruah KK, Meena RS (2018) Grain legumes: impact on soil health and agroecosystem. Springer Nature Singapore. pp. 511-539

Gogorcena Y, Iturbe-Ormaetxe I, Escuredo PR, Becana M (1995) Antioxidant defenses against activated oxygen in pea nodules subjected to water stress. Plant Physiol 108: 753–759

Gómez-Cadenas A, Mehouachi J, Tadeo F, et al. (2000) Hormonal regulation of fruitlet abscission induced by carbohydrate shortage in citrus. Planta 210: 636–643

González, E. M., Gálvez, L., Arrese-Igor, C. (2001). Abscisic acid induces a decline in nitrogen fixation that involves leghaemoglobin, but is independent of sucrose synthase activity. Journal of Experimental Botany. 52(355), 285-293

González EM, Larrainzar E, Marino D, Wienkoop S, Gil-Quintana E, Arrese-Igor C (2015) Physiological responses of N_2-fixing legumes to water limitation. In: Sulieman S, Tran LS (eds) Legume nitrogen fixation in a changing environment. Springer International Publishing, pp 5–33. https://doi.org/10.1007/978-3-319-06212-9_2

Gordon AJ, Kessler W, Minchin FR (1990) Defoliation-induced stress in nodules of white clover: I. changes in physiological parameters and protein synthesis. J Exp Bot 41: 1245-1253.

Gordon AJ, Lea PJ, Rosenberg C, Trinchant JC (2001) Nodule formation and function, in: Lea P.J., Morot-Gaudry J.F. (Eds.), Springer - Verlag Plant nitrogen, Berlin Heidelberg New York, pp. 101-146.

Graham PH, Vance CP (2000) Nitrogen fixation in perspective: an overview of research and extension needs. Field Crop Res. 65: 93-106.

Graham PH, Vance CP (2003) Legumes: importance and constraints to greater use. Plant Physiol 131: 872-877.

Gramss G, Voigt K-D, Kirsche B (199) Oxidoreductase enzymes liberated by plant roots and their effects on soil humic material. Chemosphere 38: 1481–1494

Haagenson DM, Cunningham SM, Joern BC, Volenec JJ (2003) Autumn defoliation effects on alfalfa winter survival, root physiology, and gene expression. Crop Sci 43:1340-1348.

Hamilton EW, Frank DA (2001) Can plants stimulate soil microbes and their own nutrient supply? Evidence from a grazing tolerant grass. Ecol 82: 2397-2402.

Hamilton EW, Frank DA, Hinchey PM, Murray TR (2008) Defoliation induces root exudation and triggers positive rhizospheric feedbacks in a temperate grassland. Soil Biol Biochem 40: 2865-2873.

Harasim E, Antonkiewicz J, Kwiatkowski CA (2020) The effects of catch crops and tillage systems on selected physical properties and enzymatic activity of Loess soil in a spring wheat monoculture. Agron 10: 334

Hartman K, Tringe SG (2019) Interactions between plants and soil shaping the root microbiome under abiotic stress. Biochem J 476:2705–2724. https://doi.org/10.1042/BCJ20180615

Hartwig UA, Zanetti S, Hebeisen T, Lüscher A, Frehner M, Fischer B, et al. (1996). Symbiotic nitrogen fixation: one key to understand the response of temperate grassland ecosystems to elevated CO_2? Carbon Dioxide Populations and Communities. pp. 253-264

Harvey PJ, Xiang M, Palmer JM (2002) Extracellular enzymes in the rhizosphere. Biotechnol. Bioeng 44: 1132–1139

Herrmann L, Chotte JL, Thuita M, Lesueur D (2014) Effects of cropping systems, maize residues application and N fertilization on promiscuous soybean yields and diversity of native rhizobia in Central Kenya. Pedobiologia (Jena) 57:75–85. https://doi.org/10.1016/j.pedobi.2013.12.004

Hewins DB, Fatemi F, Adams B, Carlyle C, Chang SX, Bork EW (2015) Grazing, regional climate and soil biophysical impacts on microbial enzyme activity in grassland soil of Western Canada. Pedobiologia (Jena) 58:201–209. https://doi.org/10.1016/j.pedobi.2015.10.003

Hewins DB, Broadbent T, Carlyle CN, Bork, EW (2016) Extracellular enzyme activity response to defoliation and water addition in two ecosites of the mixed grass prairie. Agri Ecosyst Envi 230: 79-86.

Hopkins A, Del Prado A (2007) Implications of climate change for grassland in Europe: impacts, adaptations and mitigation options: a review. Grass Forage Sci 62:118–126. https://doi.org/10.1111/j.1365-2494.2007.00575.x

Hu H-W, Wang J-T, Singh BK, Liu Y-R, Chen Y-L, Zhang Y-J, He J-Z (2018) Diversity of herbaceous plants and bacterial communities regulates soil resistome across forest biomes. Environ Microbiol 20: 3186-3200.

Huang X-F, Chaparro JM, Reardon KF, Zhang R, Shen Q, Vivanco JM (2014) Rhizosphere interactions: root exudates, microbes, and microbial communities. Bot 92: 267-275.

Høgh-Jensen H, Schjoerring JK (2001) Rhizodeposition of nitrogen by red clover, white clover and ryegrass leys. Soil Biol Biochem 33: 439-448.

Hossain MA, Burritt DJ, Fujita M, Munne-Bosch S, Pedro D-V (Eds.) (2017) Ascorbic acid in plant growth, development and stress tolerance. Springer Publishers.

Huber KJ, Vieira S, Sikorski J, Wüst PK, Fösel BU, Gröngröft A, Overmann J (2022) Differential response of acidobacteria to water content, soil type, and land use during an

extended drought in African savannah soils. Front Microbiol 13:750456. https://doi.org/10.3389/FMICB.2022.750456/FULL

Hubick KT, Farquhar GD, Shorter R (1986) Correlation between water-use efficiency and carbon isotope discrimination in diverse peanut (Arachis) germplasm. Aust J Plant Physiol 13:803–816

Idowu J, Ghimire R, Flynn R, Ganguli A (2020) Soil health – importance, assessment, and management. New Mexico State University. Accessed 22 January, 2021, https://aces.nmsu.edu/pubs/_circulars/CR694B/welcome.html

Iglesias DJ, Tadeo FR, Primo-Millo E, Talon M (2003) Fruit set dependence on carbohydrate availability in citrus trees. Trees Physiol 23: 199-204

Jacobi A, Katinakis P, Werner D (1994) Artificially induced senescence of soybean root nodules affects different polypeptides and nodulins in the symbiosome membrane compared to physiological ageing. J Plant Physiol 144: 533-540.

Jacques C, Girodet S, Leroy F, Pluchon S, Salon C, Prudent M (2022) Memory acclimation of water stress in pea rely on root system's plasticity and plant's ionome modulation. Front Plant Sci 13:1089720. https://doi.org/10.3389/fpls.2022.1089720

Jaleel CA, Manivannan P, Wahid A, Farooq M, Somasundaram R, Panneerselvam R (2009) Drought stress in plants: a review on morphological characteristics and pigments composition. Int J Agric Biol 11:100–105

Johnson DW, Dijkstra FA, Cheng W (2007) The effects of *Glycine max* and *Helianthus annuus* on nutrient availability in two soils. Soil Biol Biochem 39:2160–2163. https://doi.org/10.1016/j.soilbio.2007.01.036

Jones DL, Nguyen C, Finlay RD (2009) Carbon flow in the rhizosphere: carbon trading at the soil-root interface. Plant Soil 321:5–33. https://doi.org/10.1007/s11104-009-9925-0

Jones KM, Kobayashi H, Davies BW, Taga ME, Walker GC (2007) How rhizobial symbionts invade plants: the *Sinorhizobium – Medicago* model. Nat Rev 5: 619-633.

Kabiri V, Raiesi F, Ghazavi MA (2016) Tillage effects on soil microbial biomass, SOM mineralization and enzyme activity in a semi-arid calcixerepts. Agric Ecosyst Environ 232:73–84. https://doi.org/10.1016/j.agee.2016.07.022

Kaler AS, Bazzer SK, Sanz-Saez A, Ray JD, Fritschi FB, Purcell LC (2018) Carbon isotope ratio fractionation among plant tissues of soybean. Plant Phenome J 1:1–6. https://doi.org/10.2135/tppj2018.04.0002

Kang Y, An X, Ma Y, Zeng S, Jiang S, Wu W, Xie C, Wang Z, Dong C, Xu Y, Shen Q (2021) Organic amendments alleviate early defoliation and increase fruit yield by altering assembly patterns and of microbial communities and enzymatic activities in sandy pear (*Pyrus pyrifolia*). AMB Express 11: 164.

Kang Y, Han Y, Torres-Jerez I, Wang M, Tang Y, Monteros M, Udvardi M (2011) System responses to long-term drought and re-watering of two contrasting alfalfa varieties. Plant J 68:871–889. https://doi.org/10.1111/j.1365-313X.2011.04738.x

Kanté M, Riah-Anglet W, Cliquet JB, Trinsoutrot-Gattin I (2021) Soil enzyme activity and stoichiometry: linking soil microorganism resource requirement and legume carbon rhizodeposition. Agron 11:2131. https://doi.org/10.3390/agronomy11112131

Kaspar TC, Taylor HM, Shibles RM (1984) Taproot-elongation rates of soybean cultivars in the glasshouse and their relation to field rooting depth. Crop Sci 24:916–920. https://doi.org/10.2135/cropsci1984.0011183x002400050021x

Kasper S, Christoffersen B, Soti P, Racelis A (2019) Abiotic and biotic limitations to nodulation by leguminous cover crops in south Texas. Agric 9:209. https://doi.org/10.3390/agriculture9100209

Kaushal M, Wani SP (2016) Plant-growth-promoting rhizobacteria: drought stress alleviators to ameliorate crop production in drylands. Ann Microbiol 66:35–42. https://doi.org/10.1007/s13213-015-1112-3

Kavamura NV, Taketani RG, Lançoni MD, Andreote FD, Mendes R, de Melo SI (2013) Water regime influences bulk soil and rhizosphere of *Cereus jamacaru* bacterial communities in the Brazilian Caatinga biome. PLoS ONE 8:e73606. https://doi.org/10.1371/journal.pone.0073606

Keoghan JM (1982) Effects of cutting frequency and height on top-growth of pure lucerne stands. In: Langer, R.H.M. (Ed.), The Lucerne Crop. Wellington, pp. 117–128.

Kers JG, Saccenti E (2021) The power of microbiome studies: some considerations on which alpha and beta metrics to use and how to report results. Front Micro 12: 796025

Khan N, Bano A (2019) Exopolysaccharide producing rhizobacteria and their impact on growth and drought tolerance of wheat grown under rainfed conditions. PLoS ONE 14:e0222302. https://doi.org/10.1371/journal.pone.0222302

King CA, Purcell LC (2001) Soybean nodule size and relationship to nitrogen fixation response to water deficit. Crop Sci 41: 1099-1107.

King CA, Purcell LC (2005) Inhibition of N2 fixation in soybean is associated with elevated ureides and amino acids. Plant Physiol 137: 1389–1396

Köpke U, Nemecek T (2010) Ecological services of faba bean. Field Crops Res 115: 217-233.

Kondorosi E, Mergaert P, Kereszt A (2013) A paradigm for endosymbiotic life: cell differentiation of Rhizobium bacteria provoked by host plant factors. Annu Rev Microbiol 67: 611-28.

Koo B-J, Adriano DC, Bolan NS, Barton CD (2005) Root exudates and microorganisms. Encyclopedia of Soils in the Environment. 421-4 28.

Kopture S, Primoli AS, Pimienta MC (2023) Defoliation in perennial plants: predictable and surprising results in *Senna* spp. Plants 12: 587.

Kuhlgert S, Austic G, Zegarac R, Osei-Bonsu I, Hoh D, Chilvers MI, Roth MG, Bi K, TerAvest D, Weebadde P, Kuhlgert S, Austic G, Zegarac R, Osei-Bonsu I, Hoh D, Chilvers MI, Roth MG, Bi K, TerAvest D, Weebadde P, Kramer DM (2016) MultispeQ beta: a tool for large-scale plant phenotyping connected to the open photosynQ network. R Soc Open Sci 3:160592. https://doi.org/10.1098/rsos.160592

Kumar N, Srivastava P, Vishwakarma K, Kumar R, Kuppala H, Maheshwari S K, Vats S (2020) The rhizobium – plant symbiosis: state of the art. (Varma, A., Tripathi, S., Prasad, R., Ed.). Springer. pp. 1-20

Kunert KJ, Vorster BJ, Fenta BA, Kibido T, Dionisio G, Foyer CH (2016) Drought stress responses in soybean roots and nodules. Front Plant Sci 7:1015. https://doi.org/10.3389/fpls.2016.01015

Kuzyakov Y, Hill PW, Jones DL (2007) Root exudate components change residue decomposition in a simulated rhizosphere depending on temperature. Plant Soil 290: 293-305.

Kuzyakov Y, Xu X (2013) Competition between roots and microorganisms for nitrogen: mechanisms and ecological relevance. New Phytol 198: 656-669

Kwiatkowski C, Harasim E, Feledyn-Szewczyk B, Antonkiewicz J (2020) Enzymatic activity of Loess soil in organic and conventional farming systems. Agri 10: 135

Laranjo M, Alexandre A, Oliveira S (2014) Legume growth-promoting rhizobia: an overview on the *Mesorhizobium* Genus. Microbiol Res 169:2–17. https://doi.org/10.1016/j.micres.2013.09.012

Lau JA, Lennon JT, Kellogg WK, Karl DM (2012) Rapid responses of soil microorganisms improve plant fitness in novel environments. PNAS 109:14058–14062. https://doi.org/10.1073/pnas.1202319109

Lederberg J, McCray AT (2001) `Ome Sweet `Omics--A Genealogical Treasury of Words." The Scientist. 15(7, 2) p. 8.

Ledgard SF, Steele KW (1992) Biological nitrogen fixation in mixed legume/grass pastures. Plant Soil. 141(1-2), 137–153.

Lemke RL, Zhong Z, Campbell CA, Zentner R (2007) Can pulse crops play a role in mitigating greenhouse gases from North American agriculture? Agron J 99: 1719.

Lesk C, Rowhani P, Ramankutty N (2016) Influence of extreme weather disasters on global crop production. Nature 529:84–87. https://doi.org/10.1038/NATURE16467

Lesuffleur F, Paynel F, Bataillé MP, Le Deunff E, Cliquet JB (2007) Root amino acid exudation: measurement of high efflux rates of glycine and serine from six different plant species. Plant Soil 294:235–246. https://doi.org/10.1007/s11104-007-9249-x

Lesuffleur F, Salon C, Jeudy C, Cliquet JB (2013) Use of a $^{15}N_2$ labelling technique to estimate exudation by white clover and transfer to companion ryegrass of symbiotically fixed N. Plant Soil 369: 187-197

Lewin GR, Carlos C, Chevrette MG, Horn HA, McDonald BR, Stankey RJ, Fox BG, Currie CR (2016) Evolution and ecology of Actinobacteria and their bioenergy applications. Annu Rev Microbiol 70:235–254. https://doi.org/10.1146/annurev-micro-102215-095748

Li C, Hao X, Willms WD, Zhao M, Han G (2010) Effect of long-term cattle grazing on seasonal nitrogen and phosphorus concentrations in range forage species in the fescue grassland of southwestern Alberta. J Plant Nutr Soil Sci 173: 946-951.

Ling N, Wang T, Kuzyakov Y (2022) Rhizosphere bacteriome structure and functions. Nat Commun 13: 836

Liu F, Jensen CR, Andersen MN (2005) A review of drought adaptation in crop plants: changes in vegetative and reproductive physiology induced by ABA-based chemical signals. Aust J Agric Res 56:1245–1252. https://doi.org/10.1071/AR05062

Liu J, Chen J, Chen G, Guo J, Li Y (2020) Enzyme stoichiometry indicates the variation of microbial nutrient requirements at different soil depths in subtropical forests. PLoS ONE 15: e0220599.

Liyanage DK, Chathuranga I, Mori BA, Thilakarathna MS (2022) A simple, semi- automated, gravimetric method to simulate drought stress on plants. Agronomy 12:349. https://doi.org/10.3390/agronomy12020349

Liyanage DK, Torkamaneh D, Belzile F, Balasubramanian P, Hill B, Thilakarathna MS (2023) The genotypic variability among short-season soybean cultivars for nitrogen fixation under drought stress. Plants 12:1004. https://doi.org/10.3390/plants12051004

Louarn G, Pereira-Lopès E, Fustec J. et al. (2015) The amounts and dynamics of nitrogen transfer to grasses differ in alfalfa and white clover-based grass-legume mixtures as a result of rooting strategies and rhizodeposit quality. Plant Soil 389: 289–305

Lumactud RA, Dollete D, Liyanage DK, Szczyglowski K, Hill B, Thilakarathna MS (2023) The effect of drought stress on nodulation, plant growth, and nitrogen fixation in soybean during early plant growth. J Agron Crop Sci 209:345–354. https://doi.org/10.1111/jac.12627

Lynch JM, Whipps JM (1990) Substrate flow in the rhizosphere.Plant Soil 129: 1-10

Ma B, Cai Y, Bork EW, Chang SX (2018) Defoliation intensity and elevated precipitation effects on microbiome and interactome depend on site type in northern mixed-grass prairie. Soil Biol Biochem 122:163-172.

Macdonald LM, Paterson E, Dawson LA, McDonald AJS (2006) Defoliation and fertiliser influences on the soil microbial community associated with two contrasting *Lolium perenne* cultivars. Soil Biol Biochem 38: 674-682.

Maestre FT, Delgado-Baquerizo M, Jeffries TC, Eldridge DJ, Ochoa V, Gozalo B, Quero JL, García-Gómez M, Gallardo A, Ulrich W, Maestre FT, Delgado-Baquerizo M, Jeffries TC, Eldridge DJ, Ochoa V, Gozalo B, Quero JL, García-Gómez M, Gallardo A, Ulrich W, Bowker MA, Arredondo T, Barraza-Zepeda C, Bran D, Florentino A, Gaitán J, Gutiérrez JR, Huber-Sannwald E, Jankju M, Mau RL, Miriti M, Naseri K, Ospina A,

Stavi I, Wang D, Woods NN, Yuan X, Zaady E, Singh BK (2015) Increasing aridity reduces soil microbial diversity and abundance in global drylands. PNAS 112:15684–15689. https://doi.org/10.1073/pnas.1516684112

Maj D, Wielbo J, Marek-Kozaczuk M, Skorupska A (2010) Response to flavonoids as a factor influencing competitiveness and symbiotic activity of *Rhizobium leguminosarum*. Microbiol Res 165: 50-60

Mak M, Babla M, Xu SC, O'Carrigan A, Liu XH, Gong YM, Holford P, Chen ZH (2014) Leaf mesophyll K^+, H^+ and Ca^{2+} fluxes are involved in drought-induced decrease in photosynthesis and stomatal closure in soybean. Environ Exp Bot 98:1–12. https://doi.org/10.1016/j.envexpbot.2013.10.003

Manavalan LP, Guttikonda SK, Phan Tran LS, Nguyen HT (2009) Physiological and molecular approaches to improve drought resistance in soybean. Plant Cell Physiol 50:1260–1276. https://doi.org/10.1093/pcp/pcp082

Marchin RM, Ossola A, Leishman MR, Ellsworth DS (2020) A simple method for simulating drought effects on plants. Front Plant Sci 10:1715. https://doi.org/10.3389/fpls.2019.01715

Marilley L, Vogt G, Blanc M, Aragno M (1998) Bacterial diversity in the bulk soil and rhizosphere fractions of Lolium perenne and Trifolium repens as revealed by PCR restriction analysis of 16S rDNA. Plant and Soil 198: 219–224.

Marquez-Garcia B, Shaw D, Cooper JW, Karpinska B, Quain MD, Makgopa EM, Kunert K, Foyer CH (2015) Redox markers for drought-induced nodule senescence, a process occurring after drought-induced senescence of the lowest leaves in soybean (*Glycine max*). Ann Bot 116:497–510. https://doi.org/10.1093/aob/mcv030

Marriott CA, Haystead A (1990) The effect of defoliation on the nitrogen economy of white clover: regrowth and the remobilization of plant organic nitrogen. Annals Bot 66: 465-474.

Marschner P (2012) Rhizosphere Biology. Marschner's Mineral Nutrition of Higher Plants. 15, 369-388.

McNear Jr DH (2013) The rhizosphere - roots, soil and everything in Between. Nature Education Knowledge. 4(3), 1. https://www.nature.com/scitable/knowledge/library/the-rhizosphere-roots-soil-and-67500617/

McNeill AM, Zhu C, Fillery IRP (1997) Use of in situ ^{15}N-labelling to estimate the total below-ground nitrogen of pastures legumes in intact soil-plant systems. Austr J Agric Res 48: 295–304

McPherson MR, Wang P, Marsh EL, Mitchell RB, Schachtman DP (2018) Isolation and analysis of microbial communities in soil, rhizosphere, and roots in perennial grass experiments. J Vis Exp 137:e57932. https://doi.org/10.3791/57932

Meena RS, Lal R (2018) Legumes and sustainable use of soils. Springer Nature Singapore.

Meuriot F, Avice JC, Simon JC, Laine P, Decau LM, Ourry A. (2004) Influence of initial organic N reserves and residual leaf area on growth, N uptake, N partitioning and N storage in alfalfa (*Medicago sativa*) during post-cutting regrowth. Annals of Bot 94: 311-321.

Meuriot F, Decau M-L, Bertrand AM, Prud'Homme M-P, Gastal F, Simon J-C et al. (2005) Contribution of initial C and N resereves in *Medicago sativa* recovering from defoliation: impact of cutting height and residual leaf area. Funct Plant Biol 32- 321-334.

Mhadhbi H, Fotopoulos V, Mylona PV, Jebara M, Aouani ME, Polidoros AN (2011) Antioxidant gene-enzyme responses in Medicago truncatula genotypes with different degree of sensitivity to salinity. Physiol Plantarum 141: 201–214

Micheletto S, Rodriguez-Uribe L, Hernandez R, Richins RD, Curry J, O'Connell MA (2007) Comparative transcript profiling in roots of *Phaseolus acutifolius* and *P. Vulgaris* under water deficit stress. Plant Sci 173:510–520. https://doi.org/10.1016/j.plantsci.2007.08.003

Miransari M, Riahi H, Eftekhar F, Minaie A, Smith DL (2013) Improving soybean (*Glycine max* L.) N_2 fixation under stress. J Plant Growth Regul 32:909–921. https://doi.org/10.1007/s00344013-9335-7

Mndzebele B, Ncube B, Fessehazion M, Mabhaudhi T, Amoo S, du Plooy C, Venter S, Modi A (2020) Effects of cowpea-amaranth intercropping and fertiliser application on soil phosphatase activities, available soil phosphorus, and crop growth response. Agron 10: 79

Moghaddam A, Raza A, Vollmann J, Ardakani MR, Wanek W, Gollner G, Friedel JK (2013) Carbon isotope discrimination and water use efficiency relationships of alfalfa genotypes under irrigated and rain-fed organic farming. Eur J Agron 50:82–89. https://doi.org/10.1016/j.eja.2013.05.010

Moot DJ, Black AD, Lyons EM, Egan LM, Hofmann RW (2021) Pasture resilience reflects differences in root and shoot responses to defoliation, and water and nitrogen deficit. Resilient Pastures – Grassland Research and Practice Series 17: 71-80.

Mouradi M, Bouizgaren A, Farissi M, Makoudi B, Kabbadj A, Very AA, Sentenac H, Qaddoury A, Ghoulam C (2016) Osmopriming improves seeds germination, growth, antioxidant responses and membrane stability during early stage of Moroccan alfalfa populations under water deficit. Chil J Agric Res 76:265–272. https://doi.org/10.4067/S0718-58392016000300002

Mwale SE, Ochwo-Ssemakula M, Sadik K, Achola E, Okul V, Gibson P, Edema R, Singini W, Rubaihayo P (2017) Response of cowpea genotypes to drought stress in Uganda. Am J Plant Sci 8:720–733. https://doi.org/10.4236/ajps.2017.84050

Nadon B, Jackson S (2020) The polyploid origins of crop genomes and their implications: a case study in legumes. Adv Agron 159: 275-313

Näsholm T, Kielland K, Ganeteg U (2009) Uptake of organic nitrogen by plants. New Phytol 182:31–48. https://doi.org/10.1111/j.1469-8137.2008.02751.x

Naya L, Ladrera R, Ramos J, González EM, Arrese-Igor C, Minchin FR, Becana M (2007) The response of carbon metabolism and antioxidant defenses of alfalfa nodules to drought stress and to the subsequent recovery of plants. Plant Physiol 144:1104–1114. https://doi.org/10.1104/pp.107.099648

Naylor D, Coleman-Derr D (2018) Drought stress and root-associated bacterial communities. Front Plant Sci 8:2223. https://doi.org/10.3389/fpls.2017.02223

Neo HH, Layzell DB (1997) Phloem glutamine and the regulation of O2 diffusion in legume nodules. Plant Physiol 113: 259–267

Neumann G, Römheld V (2007) The release of root exudates as affected by the plant physiological status. In: Pinton, R, Zeno V, and Paolo N (eds). The rhizosphere: biochemistry and organic substances at the soil-plant interface, CRC Press, Boca Raton, FL, USA, Vol. 2, pp. 23–72.

Nishida H, Suzaki T (2018) Nitrate-mediated control of root nodule symbiosis. Curr Opin Plant Biol 44:129–136. https://doi.org/10.1016/j.pbi.2018.04.006

Niu X, Song L, Xiao Y, Ge W (2018) Drought-tolerant plant growth-promoting rhizobacteria associated with foxtail millet in a semi-arid and their potential in alleviating drought stress. Front Microbiol 8:2580. https://doi.org/10.3389/fmicb.2017.02580

Nygren P, Vaillant V, Desfontaines L, Cruz P, Domenach AM (2000) Effects of nitrogen source and defoliation on growth and biological dinitrogen fixation of Gliricidia sepius seedlings. Tree Physiol 20: 33-40.

Oldroyd GE (2013) Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. Nat Rev Microbiol 11: 252–263

Oldroyd G, Downie, J (2004) Calcium, kinases and nodulation signalling in legumes. Nat Rev Mol Cell Biol 5: 566–576

Ourry A, Kim TH, Boucaud J (1994) Nitrogen reserve mobilization during regrowth of *Medicago sativa* L. Plant Physiol 105: 831-837.

Pankhurst CE, Sprent JI (1975) Effects of water stress on the respiratory and nitrogen-fixing activity of soybean root nodules. J Exp Bot 26: 287-304.

Parida AK, Dagaonkar VS, Phalak MS, Umalkar GV, Aurangabadkar LP (2007) Alterations in photosynthetic pigments, protein and osmotic components in cotton genotypes subjected to short-term drought stress followed by recovery. Plant Biotechnol Rep 1:37–48. https://doi.org/10.1007/s11816-006-0004-1

Paynel F, Murray PJ, Cliquet JB (2001) Root exudates: a pathway for short-term N transfer from clover and ryegrass. Plant Soil 229: 235–243

Paynel F, Lesuffleur F, Bigot J, Diquélou S, Cliquet JB (2008) A study of ^{15}N transfer between legumes and grasses. Agron Sustain Dev 28:281–290. https://doi.org/10.1051/agro:2007061

Phillips DA, Fox TC, King MD, et al. (2004) Microbial products trigger amino acid exudation from plant roots. Plant Physiol 136: 2887-2894.s

Pirhofer-Walzl K, Rasmussen J, Høgh-JensenH, Eriksen J, Søegaard K, Rasmussen J (2012) Nitrogen transfer from forage legumes to nine neighbouring plants in a multi-species grassland. Plant Soil 350: 71-84.

Pohlert T (2022) PMCMRplus: calculate pairwise multiple comparisons of mean rank sums extended. R package version 1.9.6. https://CRAN.R-project.org/package=PMCMRplus. Accessed 20 Aug 2022

Poorter H, Fiorani F, Stitt M, Schurr U, Finck A, Gibon Y, Usadel B, Munns R, Atkin OK, Tardieu F, Pons TL (2012) The art of growing plants for experimental purposes: a practical guide for the plant biologist. Funct Plant Biol 39:821–838. https://doi.org/10.1071/FP12028

Popelka JC, Terryn N, Higgins TJV (2004) Gene technology for grain legumes: can it contribute to the food challenge in developing countries? Plant Sci 167: 195-206.

Preece C, Peñuelas J (2016) Rhizodeposition under drought and consequences for soil communities and ecosystem resilience. Plant Soil 409:1–7. https://doi.org/10.1007/s11104-016-3090-z

Preece C, Verbruggen E, Liu L, Weedon JT, Peñuelas J (2019) Effects of past and current drought on the composition and diversity of soil microbial communities. Soil Biol Biochem 131:28–39. https://doi.org/10.1016/j.soilbio.2018.12.022

Proulx RA, Naeve SL (2009) Pod removal, shade, and defoliation effects on soybean yield, protein, and oil. Agron J 101: 971-978.

R Core Team (2023) R: A language and environment for tatistical computing. R Foundation for Statistical Computing, Vienna. https://www.R-project.org/. Accessed 15 Mar 2023

Raeini-Sarjaz M, Barthakur NN, Arnold NP, Jones PJH (1998) Water stress, water use efficiency, carbon isotope discrimination and leaf gas exchange relationships of the bush bean. J Agron Crop Sci 180:173–179. https://doi.org/10.1111/j.1439-037x.1998.tb00387.x

Rao MA, Scelza R, Gianfreda L (2014) Soil Enzymes. Enzymes in Agricultural Sciences. OMICS Group eBooks, Foster City, pp. 10–43.

Rehman A, Nautiyal CS (2002) Effect of drought on the growth and survival of the stress-tolerant bacterium *Rhizobium* sp. NBRI2505 *sesbania* and its drought-sensitive transposon Tn5 mutant. Curr Microbiol 45:368–377. https://doi.org/10.1007/s00284-002-3770-1

Ren C, Zhao F, Shi Z, Chen J, Han X, Yang G, Feng Y, Ren G (2017) Differential responses of soil microbial biomass and carbon-degrading enzyme activities to altered precipitation. Soil Biol Biochem 115:1–10. https://doi.org/10.1016/j.soilbio.2017.08.002

Ren HB, Wei K, Jia W, Davies WJ, Zhang J (2007) Modulation of root signals in relation to stomatal sensitivity to root-sourced abscisic acid in drought-affected plants. J Integr Plant Biol 5:1410–1420

Richards JH (1993) Physiology of plants recovering from defoliation. Proc. XVII international grassland congress. Palmerston North, N.Z. p. 85-94.

Rimi F, Macolino S, Leinauer B, Lauriault LM, Ziliotto U (2014) Fall Dormancy and Harvest Stage Impact on Alfalfa Persistence in a Subtropical Climate. Agron J 106: 1258-1266.

Rochon JJ, Doyle CJ, Greef JM, Hopkins A, Molle G, Sitzia M, Scholefield D, Smith CJ (2004) Grazing legumes in Europe: a review of their status, management, benefits, research needs and future prospects. Grass Forage Sci 59:197–214

Rognes T, Flouri T, Nichols B, Quince C, Mahé F (2016) VSEARCH: a versatile open source tool for metagenomics. PeerJ 4:e2584. https://doi.org/10.7717/peerj.2584/FIG-7

Rouached A, Slama I, Zorrig W, Jdey A, Cukier C, Rabhi M, Talbi O, Limami AM, Abdelly C (2013) Differential performance of two forage species, *Medicago truncatula* and *sulla carnosa*, under water-deficit stress and recovery. Crop Pasture Sci 64:254–264. https://doi.org/10.1071/CP13049

Rubiales D, Mikic A (2015) Introduction: legumes in sustainable agriculture. CRC Crit Rev Plant Sci 34:2–3. https://doi.org/10.1080/07352689.2014.897896

Saiya-Cork KR, Sinsabaugh RL, Zak DR (2002) The effects of long term nitrogen deposition on extracellular enzyme activity in an *Acer saccharum* forest soil. Soil Biol Biochem 34:1309–1315

Sanderson MA, Brink G, Stout R, Ruth L (2013) Grass–legume proportions in forage seed mixtures and effects on herbage yield and weed abundance. Agron J 105:1289-1297.

Santos LF, Souta JF, Rocha LO, Soares CP, Santos MLC, Grativol C, et al. (2021) Altered bacteria community dominance reduces tolerance to resident fungus and seed to seedling growth performance in maize (*Zea mays* L. var. DKB 177). Microbiol Res 243: 126643

Santos-Medellín C, Liechty Z, Edwards J, Nguyen B, Huang B, Weimer BC, Sundaresan V (2021) Prolonged drought imparts lasting compositional changes to the rice root microbiome. Nat Plants 7:1065–1077. https://doi.org/10.1038/s41477-021-00967-1

Schimel JP (2018) Life in Dry soils: effects of drought on soil microbial communities and processes. Annu Rev Ecol Evol Syst 49:409–432. https://doi.org/10.1146/annurev-ecolsys-110617-062614

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537–7541. https://doi.org/10.1128/AEM.01541-09

Schwalm CR, Anderegg WRL, Michalak AM, Fisher JB, Biondi F, Koch G, Litvak M, Ogle K, Shaw JD, Wolf A, Schwalm CR, Anderegg WRL, Michalak AM, Fisher JB, Biondi F, Koch G, Litvak M, Ogle K, Shaw JD, Wolf A, Huntzinger DN, Schaefer K, Cook R, Wei Y, Fang Y, Hayes D, Huang M, Jain A, Tian H (2017) Global patterns of drought recovery. Nature 548:202–205. https://doi.org/10.1038/nature23021

Serraj R (2003) Effects of drought stress on legume symbiotic nitrogen fixation: physiological mechanisms. Indian J Exp Biol 41:1136–1141

Serraj R, Sinclair TR, Purcell LC (1999) Symbiotic N_2 fixation response to drought. J Exp Bot 50:143–155. https://doi.org/10.1093/jxb/50.331.143

Serraj R, Vadez V, Sinclair T, Sinclair TR (2001) Feedback regulation of symbiotic N_2 fixation under drought stress. Agron 21:6–7. https://doi.org/10.1051/agro:2001153ï

Sheffield J, Wood EF (2008) Projected changes in drought occurrence under future global warming from multi-model, multi-scenario, IPCC AR4 simulations. Clim Dyn 31: 79–105

Shi S, Nuccio E, Herman DJ, Rijkers R, Estera K et al. (2015) Successional trajectories of rhizosphere bacterial communities over consecutive seasons. mBio 6: e00746.

Sierra J, Daudin D, Domenach A-M, Nygren P, Desfontaines (2007) Nitrogen transfer from a legume tree to the associated grass estimated by the isotopic signature of tree root exudates: a comparison of the ^{15}N leaf feeding and natural ^{15}N abundance methods. Agron 27: 178-186

Singh G (2010) The soybean:botany,production and uses. Ed. G. Singh. Centre for Agriculture and Bioscience International.

Sita K, Sehgal A, HanumanthaRao B, Nair RM, V. Prasad PV, Kumar S, et al. (2017) Food legumes and rising temperatures: effects, adaptive functional mechanisms specific to reproductive growth stage and strategies to improve heat tolerance. Front Plant Sci 8: 16558.

Sleugh B, Moore KJ, George JR, Brummer EC (2000) Binary legume–grass mixtures improve forage yield, Quality, and seasonal distribution. Agron. J 92: 24-29.

Soumare A, Diedhiou AG, Thuita M, Hafidi M, Ouhdouch Y, Gopalakrishnan S, Kouisni L (2020) Exploiting biological nitrogen fixation: a route towards a sustainable agriculture. Plants 9:1011

Stagnari F, Maggio A, Galieni A, Pisante M (2017) Multiple benefits of legumes for agriculture sustainability: an overview. Chem. Biol Technol Agric 4: 2.

Steinauer K, Tilman D, Wragg PD, Cesarz S, Cowles JM, Pritsch K, Reich PB, Weisser WW, Eisenhauer N (2015) Plant diversity effects on soil microbial functions and enzymes are stronger than warming in a grassland experiment. Ecol 96:99–112. https://doi.org/10.1890/14-0088.1

Sulieman S, Tran LSP (2016). Legume nitrogen fixation in a changing environment: achievements and challenges. doi:10.1007/978-3-319-06212-9.

Ta TC, Faris MA (1987) Species variation in the fixation and transfer of nitrogen from legumes to associated grasses. Plant Soil 98:265–274

Tegeder M (2014) Transporters involved in source to sink partitioning of amino acids and ureides: opportunities for crop improvement. J Exp Bot 65: 1865-1878

Teixeira EI, Moot DJ, Brown HW (2007) Defoliation frequency and season affected radiation use efficiency and dry matter partitioning to roots of lucerne (*Medicago sativa* L.) crops. Europ J Agron 28: 103-111.

Tharanathan R, Mahadevamma S (2003) Grain legumes - a boon to human nutrition. Trends in Food Sci Tech 14: 507-518.

Thilakarathna MS, Cope KR (2021) Split-root assays for studying legume-rhizobia symbioses, rhizodeposition, and belowground nitrogen transfer in legumes. J Exp Bot 72:5285–5299. https://doi.org/10.1093/jxb/erab198

Thilakarathna MS, McElroy MS, Chapagain T, Papadopoulos YA, Raizada MN (2016) Belowground nitrogen transfer from legumes to non-legumes under managed herbaceous cropping systems. A review. Agron Sustain Dev 36:58. https://doi.org/10.1007/s13593-016-0396-4

Thilakarathna MS, Moroz N, Raizada MN (2017) A biosensor-based leaf punch assay for glutamine correlates to symbiotic nitrogen fixation measurements in legumes to permit rapid screening of rhizobia inoculants under control conditions. Front Plant Sci 08:1714. https://doi.org/10.3389/fpls.2017.01714

Thilakarathna MS, Raizada MN (2017) A meta-analysis of the effectiveness of diverse rhizobia inoculants on soybean traits under field conditions. Soil Biol Biochem 105:177–196. https://doi.org/10.1016/j.soilbio.2016.11.022

Thilakarathna MS, Raizada MN (2018) Visualizing glutamine accumulation in root systems involved in the legume–rhizobia symbiosis by placement on agar embedded with companion biosensor cells. Phytobiomes J 2:117–128. https://doi.org/10.1094/PBIOMES-07-18-0031-TA

Thilakarathna MS, Raizada MN (2019) A biosensor-based assay (*GlnLux*-Agar) shows defoliation triggers rapid release of glutamine from nodules and young roots of forage leguumes. Phytobiomes J 3:85–91. https://doi.org/10.1094/PBIOMES-03-19-0014-Rs

Tian K, Kong X, Yuan L, Lin H, He Z, Yao B, Ji Y, Yang J, Sun S, Tian X (2019) Priming effect of litter mineralization: the role of root exudate depends on its interactions with litter quality and soil condition. Plant Soil 440: 457-471.

Tóth Z, Táncsics A, Kriszt B, Kröel-Dulay G, Ónodi G, Hornung E (2017) Extreme effects of drought on composition of the soil bacterial community and decomposition of plant tissue. Eur J Soil Sci 68:504–513. https://doi.org/10.1111/ejss.12429

Trasar-Cepeda C, Leirós MC, Gil-Sotres F (2008) Hydrolytic enzyme activities in agricultural and forest soils. Some implications for their use as indicators of soil quality. Soil Biol Biochem 40:2146–2155. https://doi.org/10.1016/j.soilbio.2008.03.015

Trivedi P, Mattupalli C, Eversole K, Leach JE (2021) Enabling sustainable agriculture through understanding and enhancement of microbiomes. New Phytol 230: 2129–2147

Udvardi M, Poole PS (2013) Transport and metabolism in lgume rhizobia symbioses. Annu Rev Plant Biol 64:781–805. https://doi.org/10.1146/annurev-arplant

Unkovich, M., Herridge, D., Peoples, M., Cadisch, G., Boddey, B., Giller, K., et al. (2008). Measuring plant-associated nitrogen fixation in agricultural systems. Australian Centre for International Agricultural Reserach. Retreived May 13, 2021, from: https://aciar.gov.au/publication/books-and-manuals/measuring-plant-associated-nitrogen-fixation-agricultural-systems

Vadez V, Sinclair TR, Serraj R (2000) Asparagine and ureide accumulation in nodules and shoots as feedback inhibitors of N2 fixation in soybean. Physiol Plant 110: 215–223

Valentine AJ, Benedito VA, Kang Y (2011) Legume nitrogen fixation and soil abiotic stress: from physiology to genomics and beyond. In: Foyer CH, Zhang H (eds) Nitrogen metabolism in plants in the post-genomic era, vol 42, Wiley-Blackwell Publishing, pp 207–248. https://doi.org/10.1002/9781444328608.ch9

Vance CP, Heichel GH, Barnes DK, Bryan JW, Johnson LE (1979) Nitrogen fixation, nodule development, and vegetative regrowth of alfalfa (*Medicago sativa* L.) following harvest. Plant Physiol 64: 1-8.

Vance CP, Gantt JS (1992) Control of nitrogen and carbon metabolism in root-nodules. Physiol Plant 85: 266–274

Vandana UK, Rajkumari J, Singha LP, Satish L, Alavilli H, Sudheer PDVN, Chauhan S, Ratnala R, Satturu V, Mazumder PB, et al.(2021) The endophytic microbiome as a hotspot of synergistic interactions, with prospects of plant growth promotion. Biol 10: 101.

Vaseva I, Akiscan Y, Demirevska K, Anders I, Feller U (2011) Drought stress tolerance of red and white clover-comparative analysis of some chaperonins and dehydrins. Sci Hortic 130:653–659. https://doi.org/10.1016/j.scienta.2011.08.021

Voisin A-S, Guéguen J, Huyghe C, Jeuffroy M-H, Magrini M-B, et al. (2013) Legumes for feed, food, biomaterials and bioenergy in Europe: a review. Agron Sustain Dev 34:361-380

Wagner SC (2011) Biological nitrogen fixation. Nature Education Knowledge. 3(10), 15. https://www.nature.com/scitable/knowledge/library/biological-nitrogen-fixation-23570419/#

Walker TS, Bais HP, Grotewold E, Vivanco JM (2003) Root exudation and rhizosphere biology. Plant Physiol 132: 44-51.

Wang G, Bei S, Li J, Bao X, Zhang J, Schultz PA, Li H, Li L, Zhang F, et al. (2020) Soil microbial legacy drives crop diversity advantage: Linking ecological plant–soil feedback with agricultural intercropping. J Appl Ecol 58: 496-506

Wang W, Wang C, Pan D, Zhang Y, Luo B, Ji J (2018) Effects of drought stress on photosynthesis and chlorophyll fluorescence images of soybean (*Glycine max*) seedlings. Int J Agric Biol Eng 11:196–201. https://doi.org/10.25165/j.ijabe.20181102.3390

Ward NL, Challacombe JF, Janssen PH, Henrissat B, Coutinho PM, Wu M, Xie G, Haft DH, Sait M, Badger J, Ward NL, Challacombe JF, Janssen PH, Henrissat B, Coutinho PM, Wu M, Xie G, Haft DH, Sait M, Badger J, Barabote RD, Bradley B, Brettin TS, Brinkac LM, Bruce D, Creasy T, Daugherty SC, Davidsen TM, DeBoy RT, Detter JC, Dodson RJ, Durkin AS, Ganapathy A, Gwinn-Giglio M, Han CS, Khouri H, Kiss H, Kothari SP, Madupu R, Nelson KE, Nelson WC, Paulsen I, Penn K, Ren Q, Rosovitz MJ, Selengut JD, Shrivastava S, Sullivan SA, Tapia R, Thompson LS, Watkins KL, Yang Qi, Yu C, Zafar N, Zhou L, Kuske CR (2009) Three genomes from the phylum Acidobacteria provide insight into the lifestyles of these microorganisms in soils. Appl Environ Microbiol 75:2046–2056. https://doi.org/10.1128/AEM.02294-08

Weiss S, Xu ZZ, Peddada S, Amir A, Bittinger K, Gonzalez A, Lozupone C, Zaneveld JR, Vázquez-Baeza Y, Weiss S, Xu ZZ, Peddada S, Amir A, Bittinger K, Gonzalez A, Lozupone C, Zaneveld JR, Vázquez-Baeza Y, Birmingham A, Hyde ER, Knight R (2017) Normalization and microbial differential abundance strategies depend upon data characteristics. Microbiome 5:27. https://doi.org/10.1186/s40168-017-0237-y

Weston LA, Ryan PR, Watt M (2012) Mechanisms for cellular transport and release of allelochemicals from plant roots into the rhizosphere. J Exp Bot 63: 3445–3454

White J, Prell J, James EK, Poole P (2007) Nutrient sharing between symbionts. Plant Physiol 144: 604–614

Wichern F, Eberhardt E, Mayer J, Joergensen RG, Müller T (2008) Nitrogen rhizodeposition in agricultural crops: methods, estimates and future prospects. Soil Biol Biochem 40:30–48. https://doi.org/10.1016/j.soilbio.2007.08.010

Wilson CH, Strickland MS, Hutchings JA, Bianchi TS, Flory SL (2018) Grazing enhances belowground carbon allocation, microbial biomass, and soil carbon in a subtropical grassland. Glob. Change Biol. 24: 2997-3009.

Xu F, Guo W, Wang R, Xu W, Du N, Wang Y (2009) Leaf movement and photosynthetic plasticity of black locust (*Robinia pseudoacacia*) alleviate stress under different light and water conditions. Acta Physiol Plant 31:553-563

Xu L, Naylor D, Dong Z, Simmons T, Pierroz G, Hixson KK, Kim YM, Zink EM, Engbrecht KM, Wang Y, Xu L, Naylor D, Dong Z, Simmons T, Pierroz G, Hixson KK, Kim Y-M, Zink EM, Engbrecht KM, Wang Yi, Gao C, DeGraaf S, Madera MA, Sievert JA, Hollingsworth J, Birdseye D, Scheller HV, Hutmacher R, Dahlberg J, Jansson C, Taylor JW, Lemaux PG, Coleman-Derr D (2018) Drought delays development of the sorghum root microbiome and enriches for monoderm bacteria. PNAS 115:E4284–E4293. https://doi.org/10.1073/PNAS.1717308115

Xu X, Sherry RA, Niu S, Li D, Luo Y (2013) Net primary productivity and rain-use efficiency as affected by warming, altered precipitation, and clipping in a mixed-grass prairie. Global Change Biol 19: 2753-2764.

Xun W, Zhao J, Xue C, Zhang G, Ran W, Wang B, Shen Q, Zhang R (2016) Significant alteration of soil bacterial communities and organic carbon decomposition by different long-term fertilization management condition of extremely low productivity arable soil in South China. Envi Micro 18: 1907-1917

Yuste JC, Fernandez-Gonzales AJ, Fernandez-Lopez M, Ogaya R, Penuelas J, Sardans J, Lloret F (2014) Strong functional stability of soil microbial communities under semiarid Mediterranean conditions and subjected to long-term shifts in baseline precipitation. Soil Biol Biochem 69:223–233. https://doi.org/10.1016/j.soilbio.2013.10.045

Zahran HH (1999) Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. Microbiol Mol Biol Rev 63:968–989. https://doi.org/10.1128/mmbr.63.4.968-989.1999

Zargar SM, Gupta N, Nazir M, Mahajan R, Malik FA, Sofi NR, Shikari AB, Salgotra RK (2017) Impact of drought on photosynthesis: molecular perspective. Plant Gene 11:154–159. https://doi.org/10.1016/j.plgene.2017.04.003

Zhang C, Shi S, Liu Z, Yang F, Yin G (2018) Drought tolerance in alfalfa (*Medicago sativa* L.) varieties is associated with enhanced antioxidative protection and declined lipid peroxidation. J Plant Physiol 232:226–240. https://doi.org/10.1016/j.jplph.2018.10.023

Zhang Y, Lu W, Zhang H, Zhou J, Shen Y (2018) Grassland management practices in Chinese steppes impact productivity, diversity and the relationship. Front Agron Sci Eng 5: 57-63.

Appendix

Appendix – A

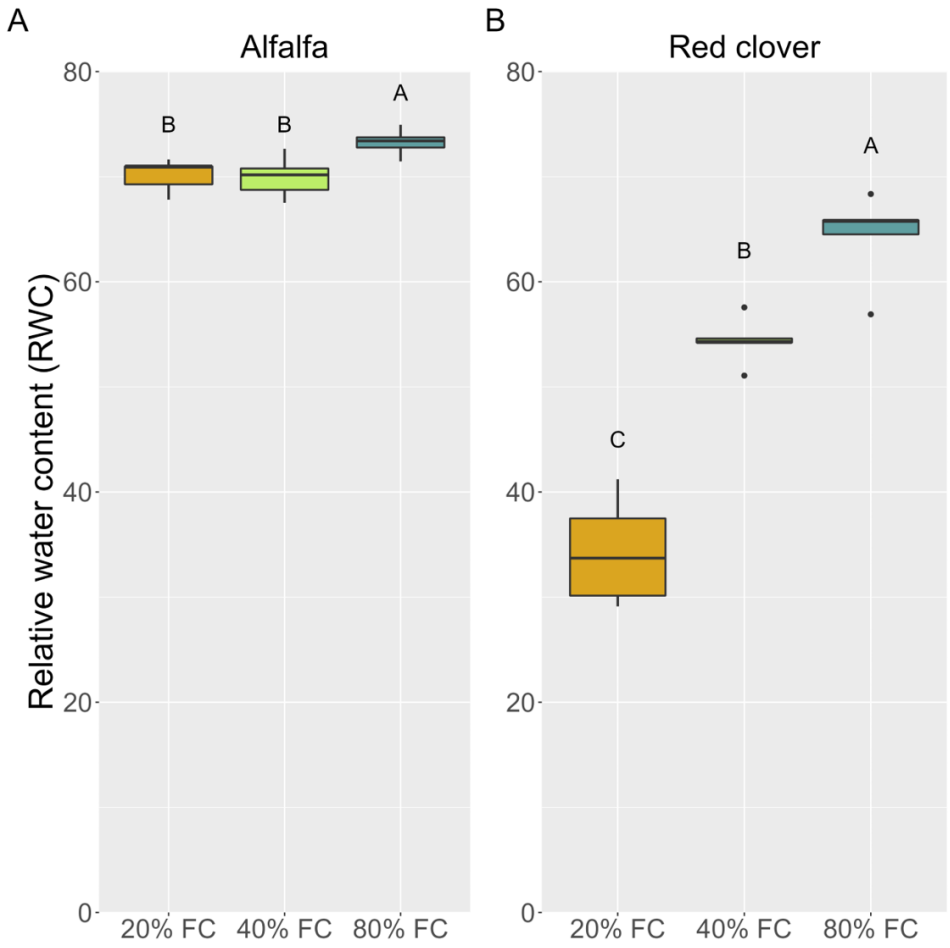


Figure A.1. Effects of three weeks of moderate and severe drought stress on shoot relative water content in alfalfa and red clover plants.

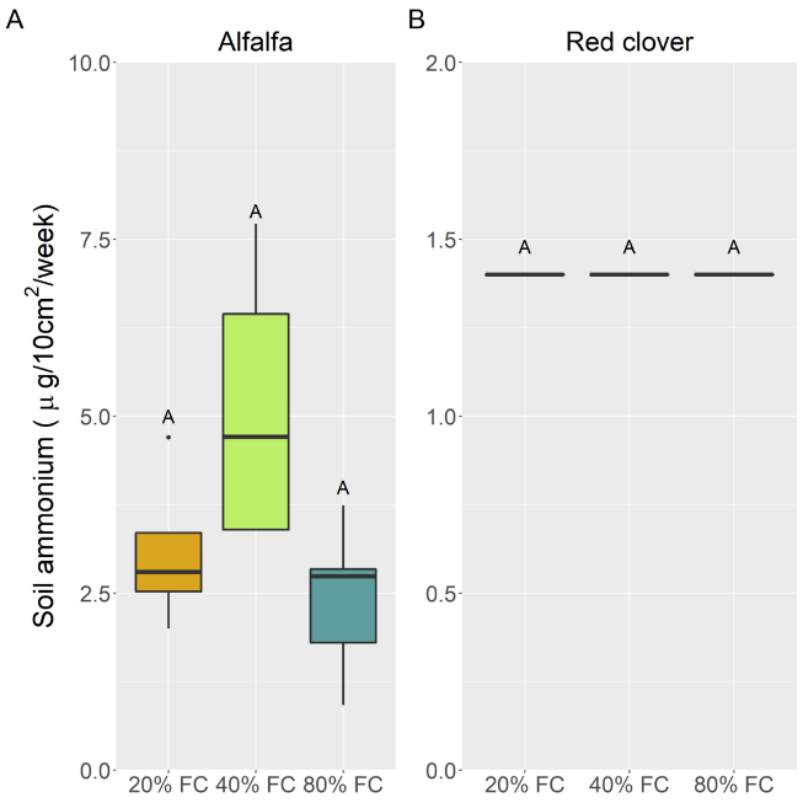


Figure A.2. Effects of three weeks of moderate and severe drought stress on soil available ammonium in alfalfa and red clover soils

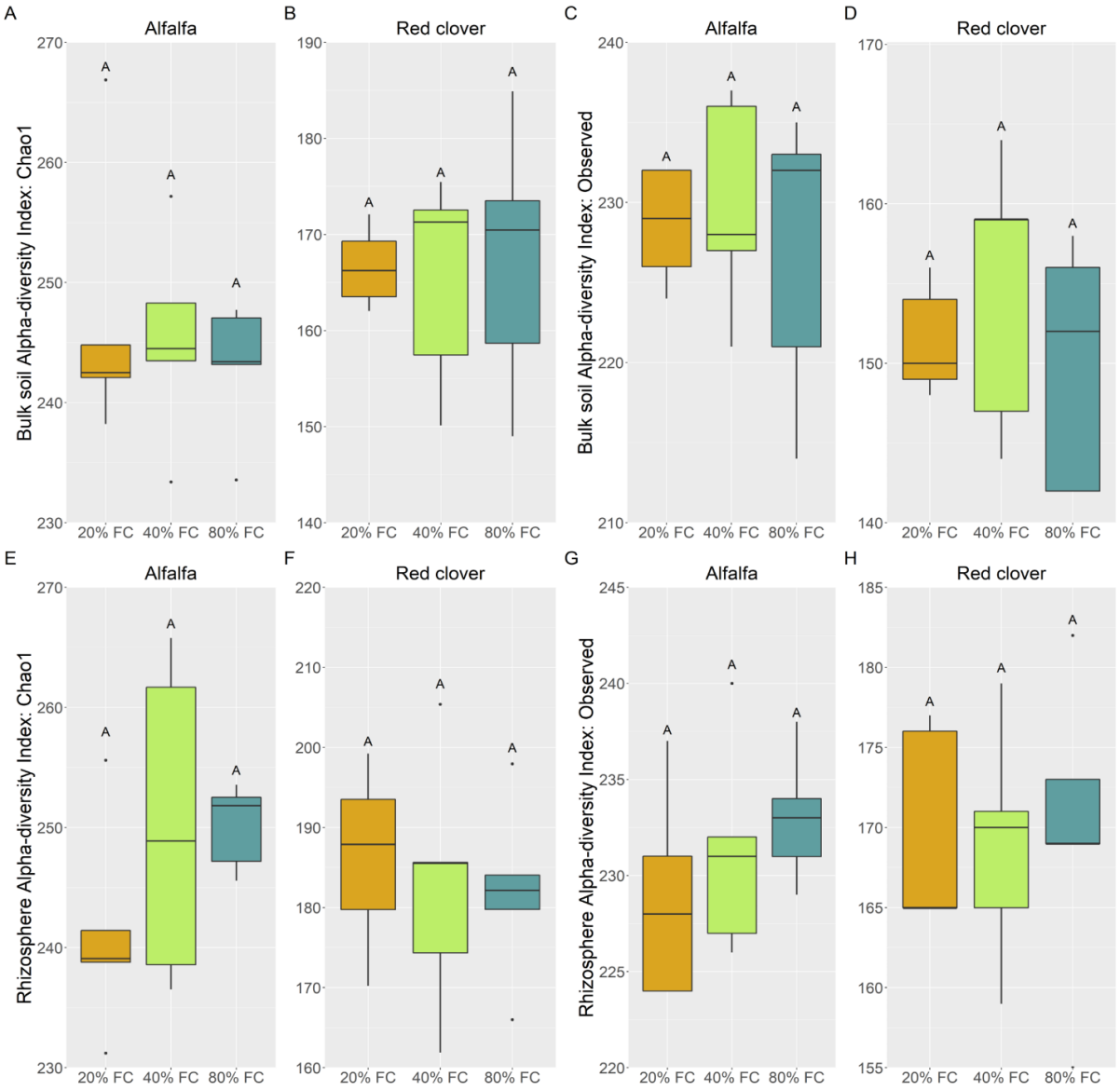


Figure A.3. Alpha diversity analysis of alfalfa and red clover bulk soil and rhizosphere under moderate (40% field capacity) and severe (20% field capacity) drought conditions reflected by Chao1 and Observed diversity indices

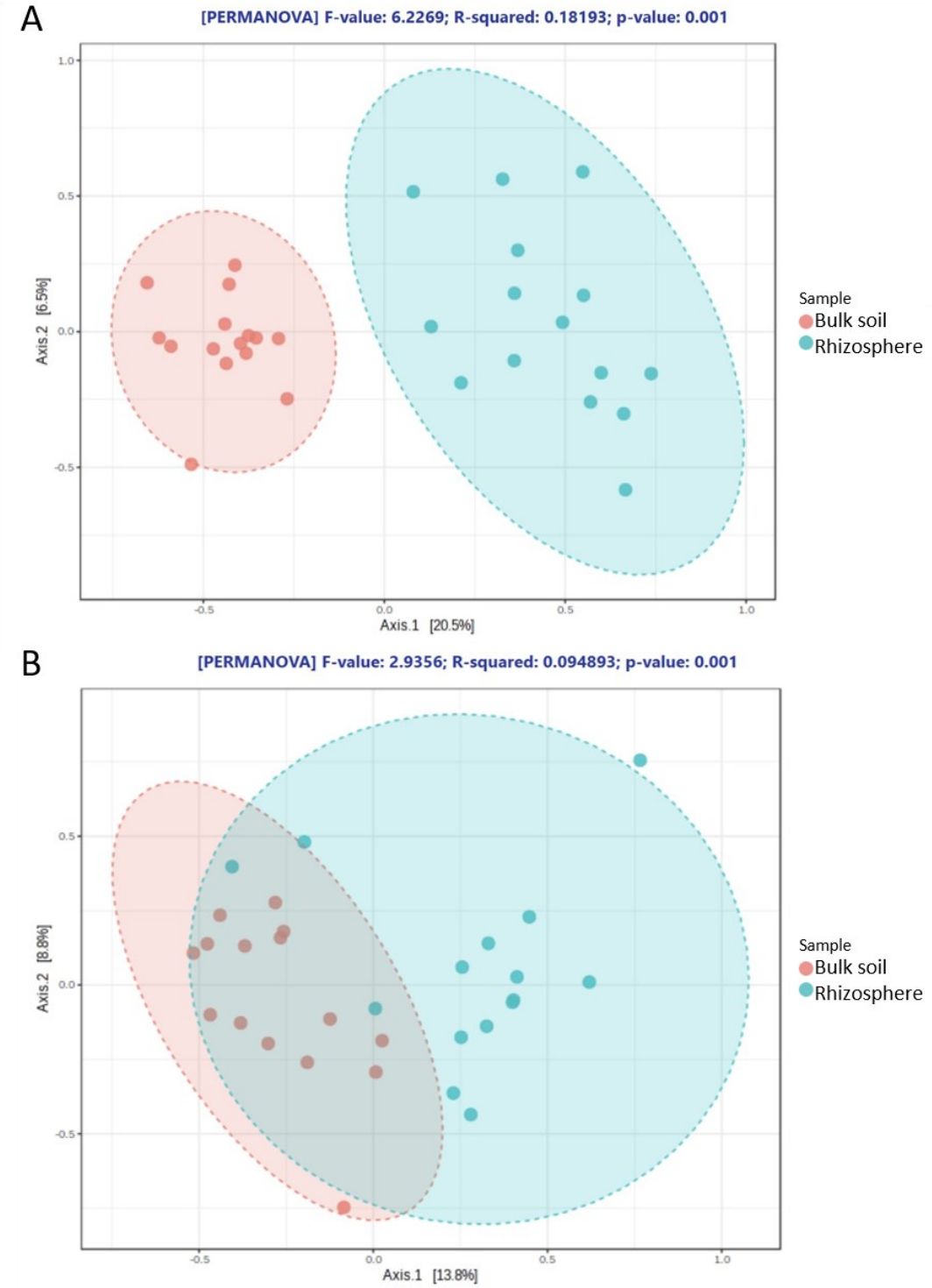


Figure A.4. Beta-diversity of alfalfa and red clover bulk and rhizosphere soils under drought treatment.

Appendix – B

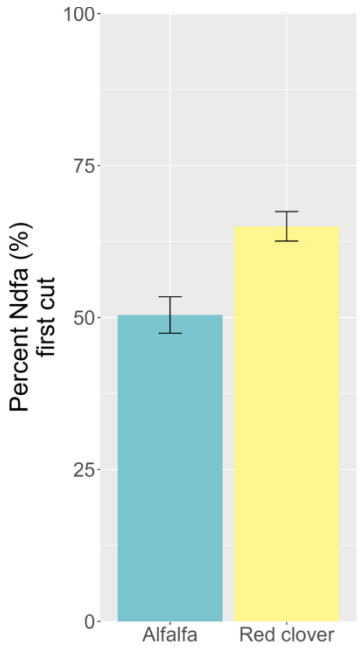


Figure B.1. Average percentage of nitrogen derived from the atmosphere (%Ndfa) in shoot samples of alfalfa and red clover at first cut.

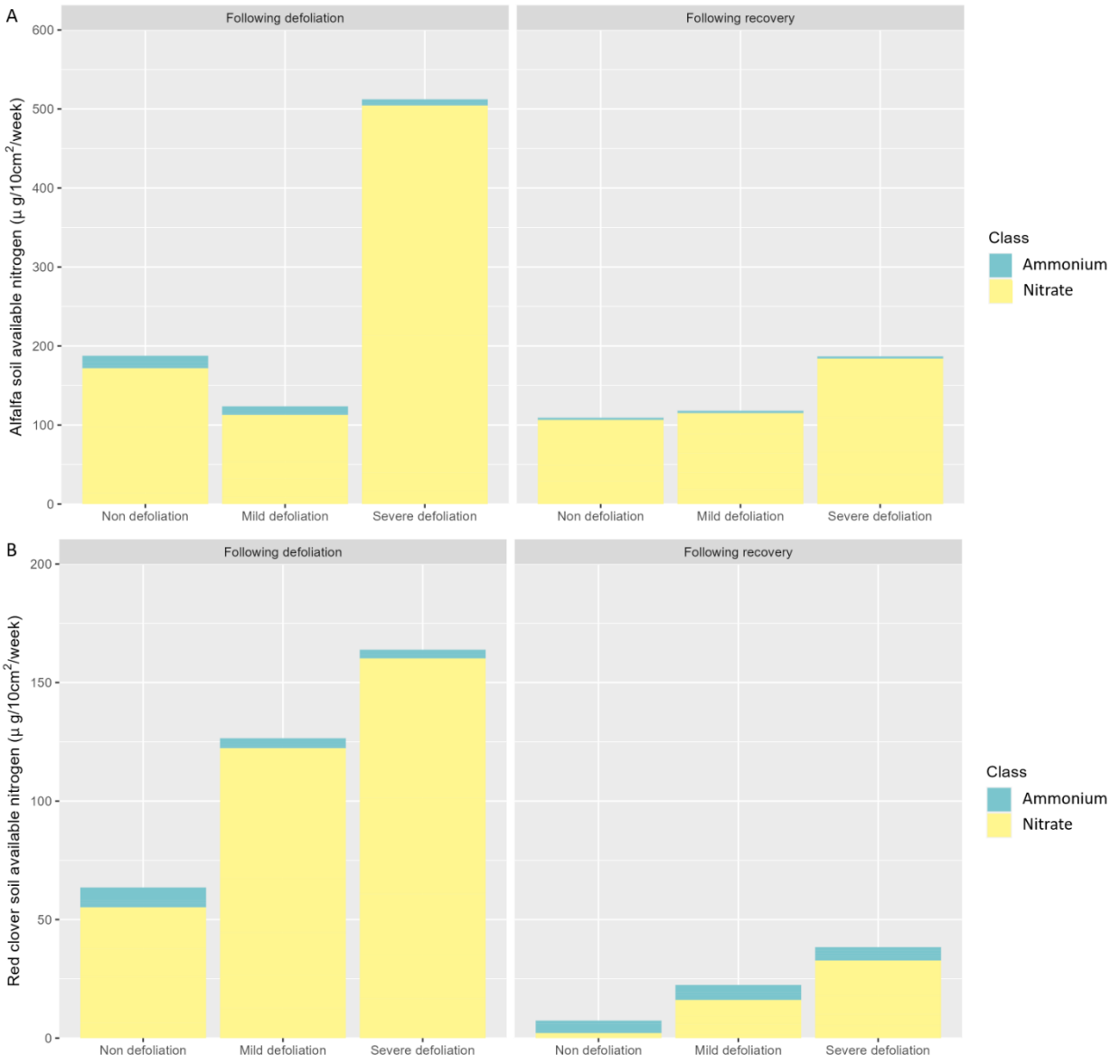


Figure B.2. The effects of mild (50%) and severe defoliation (100%) on soil available nitrogen forms (NO_3^- and NH_4^+) following one week of defoliation and four weeks of recovery. (A) Alfalfa. (B) Red clover. The soil available NO_3^- and NH_4^+ were measured using the plant root simulator (PRS) probes.

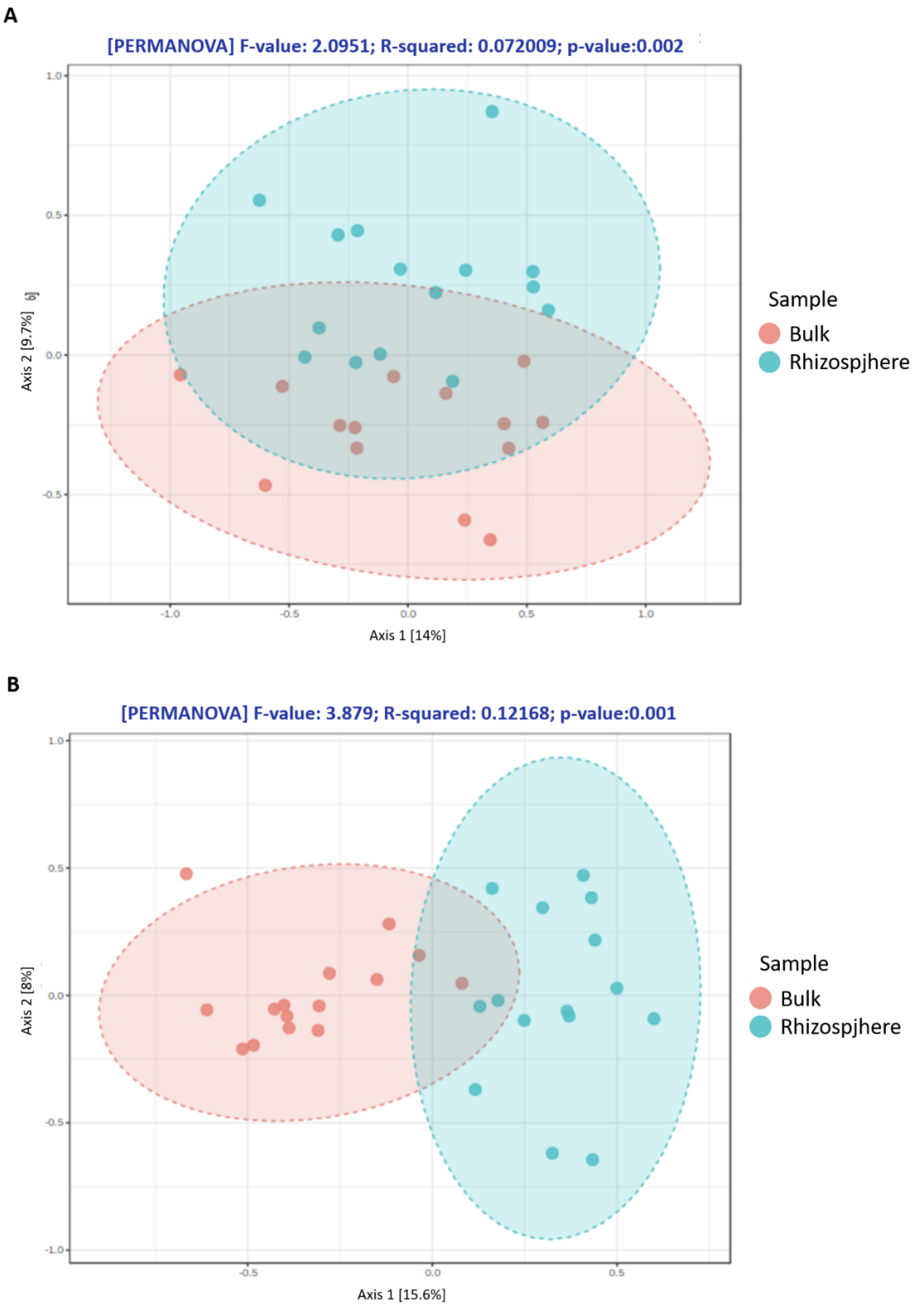


Figure B.3. Beta-diversity of alfalfa and red clover bulk and rhizosphere soils following defoliation treatment.