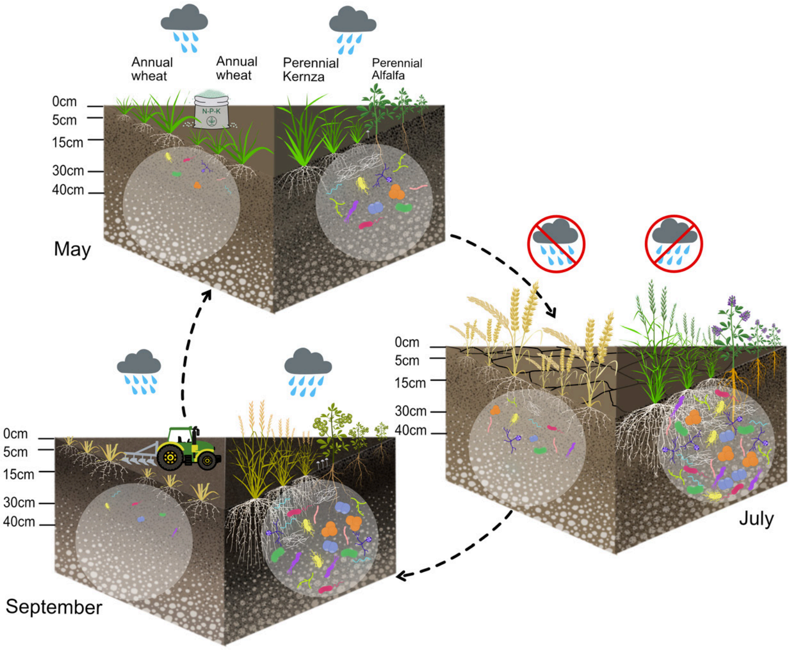
ACTA UNIVERSITATIS AGRICULTURAE SUECIAE

DOCTORAL THESIS NO. 2024:92
FACULTY OF LANDSCAPE ARCHITECTURE, HORTICULTURE
AND CROP PRODUCTION SCIENCE

Intercropping perennial cereal and legumes for improving biological soil health and microbial drought resilience

SHOUJIAO LI



Intercropping perennial cereal and legumes for improving biological soil health and microbial drought resilience

Shoujiao Li
Faculty of Landscape Architecture, Horticulture and Crop Production Sciences
Department of Biosystems and Technology
Alnarp

SLU
SWEDISH UNIVERSITY
OF AGRICULTURAL
SCIENCES

DOCTORAL THESIS
Alnarp 2024

Acta Universitatis Agriculturae Sueciae
2024:92

Cover: Perennial Kernza-alfalfa intercrops support greater numbers of soil microbes than annual wheat, even under drought conditions. © 2024 by Shoujiao Li is licensed under CC BY-NC-SA 4.0)

ISSN 1652-6880
ISBN (print version) 978-91-8046-383-6
ISBN (electronic version) 978-91-8046-419-2
https://doi.org/10.54612/a.4ve4ma46ur

Swedish University of Agricultural Sciences, Department of Biosystems and Technology , Alnarp, Sweden

Print: SLU Grafisk service, Alnarp 2024

Intercropping perennial cereal and legumes for improving biological soil health and microbial drought resilience

Abstract

Conventional annual cereal production relies on synthetic inputs and intensive agronomic practices that compromise soil health and increase vulnerability to drought. In contrast, perennial cereals such as intermediate wheatgrass (IWG) provide continuous soil cover and deep root systems for several years, making them promising for improving soil health and drought resilience. Soil microbes, particularly arbuscular mycorrhizal (AM) fungi, play a critical role in plant growth and drought resistance. Intercropping IWG with legumes may support beneficial soil microbes during drought and further improve soil health by increasing long-term crop diversity. However, the effects of IWG on soil microbial communities and their responses to drought stress remain poorly understood. This thesis investigates the interactions among IWG, legumes, and soil microbes under drought conditions. Controlled pot and field experiments were conducted to assess the yield and interspecific interactions in IWG-legume intercropping. The results showed that intercropping maintains or increases IWG grain yield and nitrogen (N) content, particularly under N-limited or drought conditions, due to the complementary use of N. A simulated drought experiment using *in-situ* rainfall reduction and phospholipid fatty acid analysis demonstrated the importance of soil microbial community composition and AM fungal biomass in maintaining the grain yield and N content of IWG under drought. IWG cropping shifted microbial communities towards a higher abundance of fungi and AM fungi and increased drought resilience. Furthermore, IWG increased soil total carbon in the upper soil layers. Overall, this thesis highlights the potential of perennial cereal-legume intercropping to improve soil biological health, enhance microbial drought resilience and stabilise grain yield, making it a promising strategy for climate change adaptation and mitigation.

Keywords: drought, intercropping, intermediate wheatgrass, microbial biomass, microbial community, perennial, PLFA, rainfall reduction, resistance and resilience

Samodling av fleråriga spannmål och baljväxter för förbättrad biologisk jordhälsa och återhämtningsförmåga från torka

Sammanfattning

Konventionell produktion av ettåriga spannmålsgrödor bygger på användning av syntetiska gödselmedel och intensiv jordbearbetning, vilket påverkar jordhälsan negativt och ökar sårbarheten för torka. Däremot ger den fleråriga spannmålsgrödan *Thinopyrum intermedium* (IWG) ett kontinuerligt jordtäcke och djupa rotsystem under flera år, vilket gör dem lovande för att förbättra jordhälsan och motståndskraften mot torka. Markmikrober, särskilt arbuskulära mykorrhiza-svampar, spelar en avgörande roll för grödors tillväxt och motståndskraft mot torka. Samodling av IWG med baljväxter kan stödja gynnsamma jordmikrober under torka och ytterligare förbättra jordhälsan. Dock saknas fortfarande kunskap om effekterna av IWG på mikrobiella samhällen i marken och deras svar på torkstress. Kontrollerade kruk- och fältexperiment genomfördes för att bedöma avkastning och interspecifika interaktioner mellan samodling av IWG och baljväxter. Resultaten visar att samodling bibehåller eller ökar IWG kornutbyte och kväveinnehåll (N), särskilt under N-begränsade eller torra förhållanden, på grund av den kompletterande användningen av N. Ett simulerat torkexperiment med minskad nederbörd och fosfolipidfettsyraanalys visade vikten av sammansättningen i de mikrobiella samhällena och mykorrhiza-svamparnas biomassa för att upprätthålla kornutbytet och N-innehållet i IWG under torka. I IWG-odlingssystemen förändrades de mikrobiella samhällena mot en högre förekomst av svampar och mykorrhiza-svampar och ökade den mikrobiella biomassans återhämtningsförmåga från torka. Dessutom ökade IWG också den totala mängden kol i de övre jordlagren. Sammantaget belyser denna avhandling potentialen för flerårig samodling av spannmål och baljväxter för att förbättra jordens biologiska hälsa, stärka mikrobiell återhämtningsförmåga från torka och stabilisera spannmålsavkastningen, vilket gör det till en lovande strategi för anpassning till och begränsning av klimatförändringar.

Nyckelord: mikrobiell biomassa, mikrobiella samhällen, minskning av nederbörd, motståndskraft mot torka, perenn spannmål, PLFA, samodling, *Thinopyrum intermedium,* torka, återhämtningsförmåga från torka

多年生禾本科与豆科作物间作有利于增强土壤(生物性)健康和微生物的抗旱性

摘要

全球气候变化加剧了干旱对一年生谷物的生长造成的显著不利影响。由于过度依赖农药和化肥的使用以及对土壤的频繁耕作，一年生谷物的栽培也正是造成气候变化的原因之一。而多年生谷物比如中间型偃麦草，能够生长并覆盖土壤多年，具有发达的根系结构，能够改善土壤健康，并增强对气候变化的适应能力。土壤微生物在促进作物生长和适应干旱胁迫以及提升土壤健康方面起着关键作用。而将多年生谷物与豆科间作可能会进一步提升环境效益和对气候变化的适应能力。因为多年生豆禾间作长期地增加了作物多样性。目前关于新型多年生谷物如何影响土壤微生物群落，以及它们如何响应干旱胁迫还没有充分的研究。本论文主要探究了间作系统中干旱条件下，中间型偃麦草、豆科作物、土壤微生物三者之间的相互作用。首先，本论文在盆栽和田间实验中研究了中间型偃麦草和豆科间作的产量优势和种间相互作用。研究发现，由于不同氮源的补偿性利用和氮素转移，中间型偃麦草和豆科间作后具有互利效应，表现在产量和氮素累积优势。在盆栽实验低氮肥处理以及田间试验干旱条件下，这种间作优势更为显著。然而，土壤微生物对这一现象的贡献并不清楚。本论文通过在多年生和一年生作物系统中，田间减少降雨而模拟了干旱，并使用了酸性磷脂脂肪酸法研究土壤微生物对干旱胁迫的响应，以及对上述间作优势的潜在贡献。结果表明，间作系统中土壤微生物群落组成和丛枝菌根真菌生物量，对中间型偃麦草在干旱条件下维持产量和氮含量稳定至关重要。中间型偃麦草和紫花苜蓿间作后，土壤微生物群落转变为更富含真菌和丛枝菌根真菌的状态，而使得微生物群落的整体抗旱性更强。土壤再湿润后，间作系统中土壤微生物的生物量对干旱的恢复更快。此外，中间型偃麦草和苜蓿间作还增加了上层土壤中碳的含量。本论文表明，多年生谷物与豆科作物间作能够提升土壤微生物的抗旱性、改善土壤健康状况、并在干旱条件下稳定谷物产量，使其更有效地适应气候变化。

关键词: 多年生, 中间型偃麦草, 间作, 干旱, 抗旱性, 微生物生物量, 微生物群落, 酸性磷脂脂肪酸法, 就地降雨减少

Dedication

To my family

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Li, S.**, Jensen, E.S., Liu, N., Zhang, Y., Dimitrova Mårtensson, L.-M. (2021). Species Interactions and Nitrogen Use during Early Intercropping of Intermediate Wheatgrass with a White Clover Service Crop. Agronomy, 11 (2), pp. 388. https://doi.org/10.3390/agronomy11020388

II. Dimitrova Mårtensson, L.-M., Barreiro, A., **Li, S.,** Jensen, E.S. (2022). Agronomic performance, nitrogen acquisition and water-use efficiency of the perennial grain crop *Thinopyrum intermedium* in a monoculture and intercropped with alfalfa in Scandinavia. Agronomy for Sustainable Development. 42 (21). https://doi.org/10.1007/s13593-022-00752-0

III. **Li, S.,** Barreiro, A., Almeida, J. P., Prade, T., Dimitrova Mårtensson, L.-M. (2025). Perennial crops shape the soil microbial community and increase the soil carbon in the upper soil layer. Soil Biology and Biochemistry. 200 (109621). https://doi.org/10.1016/j.soilbio.2024.109621

IV. **Li, S.,** Almeida, J. P., Barreiro, A., Prade, T., Dimitrova Mårtensson, L.-M. (2024). Perennial crops foster drought resistant and resilient soil microbes and sustain grain yield under rainfall reduction. (Manuscript)

All published papers are reproduced with the permission of the publishers.

The contribution of Shoujiao Li to the papers included in this thesis was as follows:

I. SL conceived and planned the experiment, carried out the experiment, collected data, performed data analyses, wrote the original manuscript, edited the manuscript at different stages, and communicated with the publisher, editor, and reviewers.

II. SL carried out sample preparation for laboratory analyses, contributed to the interpretation of results, and provided feedback on the manuscript.

III. SL contributed to the experimental design, organized and conducted plant and soil sampling in the field, performed laboratory measurements, conducted data analyses and visualization, interpreted the results, wrote the entire original manuscript, edited the manuscript at different stages, communicated with the editor and reviewers.

IV. SL coordinated and carried out plant and soil sampling in the field, conducted laboratory measurements, performed data visualization and analyses, and drafted the entire original manuscript.

Other publications not included in this thesis:

Li, S., Barreiro, A., Jensen, E.S., Zhang, Y., Dimitrova Mårtensson, L.-M. (2019). Early interspecific dynamics, dry matter production and nitrogen use in Kernza (*Thinopyrum intermedium*) – alfalfa (*Medicago sativa* L.) mixed intercropping. Acta Agriculturae Scandinavica, Section B — Soil & Plant Science, 70 (2), pp. 165-175. https://doi.org/10.1080/09064710.2019.1686164

List of tables

List of figures

Abbreviations

Abbreviation	**Meaning**
^{13}C	A heavier stable isotope of carbon
^{15}N	A heavier stable isotope of nitrogen
AM	Arbuscular Mycorrhizal
ANOVA	Analysis of Variance
C	Carbon
CON	Conventional
DNA	Deoxyribonucleic Acid
IWG	Intermediate Wheatgrass
N	Nitrogen
NLFA	Neutral Lipid Fatty Acid
PLFA	Phospholipid Fatty Acid
qPCR	real-time quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
SAFE	Swedish Infrastructure for Ecosystem Science's Agroecological Field Experiment
SDGs	The United Nations Sustainable Development Goals
SITES	Swedish Infrastructure for Ecosystem Science
$\Delta^{13}C$	The discrimination against ^{13}C during photosynthesis

1. Introduction

1.1 Climate change pressures on agricultural crop production

Sustainable agriculture aims to ensure future food security, environmental health, economic profitability, and social equity (Allen et al. 1991). However, agricultural production and sustainability are threatened by various natural and anthropogenic factors, including climate change (Dubey et al. 2020). The consequences of climate change, including global warming, drought, and changes in precipitation patterns, add more uncertainty and complexity to agricultural practice and thus demand more efforts to increase sustainability. The impact of climate change on crop yields is expected to vary across regions. In some areas, crop yields may increase, while in others they may decrease, depending on factors such as latitude and irrigation practices (Kang et al. 2009). In northern Europe, the dry summer of 2018 provided an example of the negative impacts of extreme climate events on crop yields and the vulnerability of current agriculture. In Sweden, cereal yields were reduced by up to 50% and livestock numbers were reduced due to a lack of affordable fodder and feed in 2018 (Grusson et al. 2021a). By 2050, the average daily air temperature in Sweden is predicted to increase 1-2 °C overall (Grusson et al. 2021b), and that the annual precipitation is predicted to change (decrease/increase) in a range from 15.9% to 25.2% in southern Sweden (Olsson et al. 2016). In consequence, water availability is expected to decrease due to increasing temperatures and high precipitation variability which could ultimately lead to lower crop production (Kang et al. 2009). Therefore, there is an urgent need to study the effects of climate-change-induced drought on crop production in order to develop a more climate-resilient and sustainable agriculture.

1.2 Conventional monoculture of annual crops impairs long-term sustainability

In addition to climate change, another major challenge facing agriculture is providing enough food for a growing population. The world population reached 8 billion in 2022, and is projected to reach 9.7 billion by 2050 (Jain et al. 2023). Population growth is expected to increase global food demand by 35% to 56% between 2010 and 2050 (van Dijk et al. 2021). Moreover, current agricultural food systems are responsible for a number of environmental problems. In 2015, around 34% of total anthropogenic greenhouse gas emissions came from agricultural food systems (Crippa et al. 2021). In 2010, 57% global greenhouse gas emissions from animal-based foods (including livestock feed) and 29% from plant-based foods (Xu et al. 2021). Current agricultural practices are causing other serious problems such as biodiversity loss (Tsiafouli et al. 2015), soil degradation, soil erosion, and water resource depletion. They are major drivers of transgressing the planetary boundaries of biosphere integrity and altering biogeochemical fluxes, thus destabilising the Earth system at planetary scales (Campbell et al. 2017). This raises concerns about how to sustainably feed a growing population without further exacerbating environmental degradation and the climate crisis.

Agriculture occupies 40% of the Earth’s land surface (Ramankutty et al. 2008), and about 70% of the world’s arable land is dominated by annual grain crops (Pimentel et al. 2012). These crops are often grown in simple crop rotations based on a few crop species and rely heavily on fertiliser, pesticides, water, energy inputs and intensive soil tillage. Annual crop monoculture has the advantage of high yields, but comes at the expense of the environment and ecosystem health. Food products from conventional agriculture come at a low price but do not take into account the hidden costs of negative impacts on soil, animal, human and environmental health. With these and other known impacts, conventional annual crop monoculture is not sustainable and contradictory with several sustainable development goals (SDGs). A transition from the business-as-usual agricultural production to sustainable agro-food systems is urgently needed to develop an agriculture that can address both production and environmental challenges (Testa et al. 2022), produce healthy food (SDG2 and 3), protect ground and surface water quality (SDG6), mitigate climate change (SDG13), avoid soil degradation, and support biodiversity (SDG15) (Bouma et al. 2021). Agroecology is a promising approach for achieving such transition. Agroecology is not solely a set of sustainable agronomic practices but a merging of approaches (science, practices, and movements) to achieve sustainable, equitable, and fair food systems, while respecting ecological principles (Sachet et al. 2021). Agro-ecological transition calls for the complete transformation of our food systems.

1.3 Agro-ecological transitions are essential to address environmental challenges

1.3.1 How do agro-ecological transitions happen?

Agro-ecological transitions can occur at different levels from farm to table. The studies in this thesis mainly focused on the transitions at farm level. According to Gliessman (2016), changes at farm have three levels; (1) improving resource use efficiency and reducing industrial inputs through practices such as precision agriculture (i.e. site-specific management); (2) implementing alternative practices for synthetic inputs through such as organic farming, using N-fixing cover crops and organic composts; (3) redesigning and rediversifying production systems through such as agroforestry, rotations, and integrating animals with crop production. Changes at level three can implement fundamental changes and address the root causes of many environmental problems (Gliessman 2016). There is an urgent need to redesign cropping systems and investigate alternative agronomic practices to further the transition towards more sustainable systems at the farm level.

1.3.2 Alternative practices and cropping system redesign

Although there is no silver bullet for achieving agro-ecological transitions at farm level, certain practices like crop diversification, nutrient and soil management, crop rotation, cover crops, no-till and low-till farming, integrated pest management, and rotational grazing, have been recognised to enhance sustainability (Horrigan et al. 2002). One of these, crop diversification is considered a key pillar for agro-ecological transition (Alletto et al. 2022). Crop diversification, for example, through legume and cereal intercropping, i.e. the simultaneous growing of two or more crop species on the same area (Willey 1979), has been shown to reduce global demand for synthetic N fertilisers (Jensen et al. 2020) and improve soil fertility through biological N fixation by legumes. In addition, intercropping can improve nutrient use efficiency, increase yield stability and yield per unit area, and reduce pest incidence compared to monoculture, showing great potential to support the development of a more sustainable cropping system (Bedoussac et al. 2015). However, intercropping of cereals and legumes is not yet well implemented due to the increased complexity in management, mechanical implementation, development of cultivars for intercropping etc. (Martin-Guay et al. 2018).

In addition to intercropping practices, a growing body of research has recently sought a way to improve sustainability through the development of perennial grain cropping systems (Glover 2005; Culman et al. 2013; Soto-Gómez & Pérez-Rodríguez 2022). As perennial grain crops can live and remain productive for two or more years without the need for annual replanting, they hold great promise for reducing the labour requirements, inputs and negative impacts of tillage, as well as supporting agro-ecological principles. Perennial grain crops therefore provide an interesting option for cropping system redesign in the context of agro-ecological transitions.

1.4 Perennial grain crops support agro-ecological transitions

1.4.1 Potential of perennial grain crops for cropping system redesign

Recent research has demonstrated the ability of perennial grain crops to provide ecosystem services, i.e. the benefits, goods and services that the human population derives directly or indirectly from ecosystem functions and the contributions that ecosystems make to human well-being (Costanza et al. 1997; Haines-Young & Potschin 2010). Perennial grain crops have been shown to reduce nitrate leaching, protect water quality, improve wildlife habitat, sequester C and mitigate climate change (DeHaan et al. 2023). In particular, a nine-year field study (Shang et al. 2024) showed that three perennial grain cropping systems increased topsoil C and N stocks by an average of 1.4 Mg C ha^{-1} and 0.18 Mg N ha^{-1} over time, respectively, while two annual cropping systems reduced topsoil C and N stocks by an average of 3.4 Mg C ha^{-1} and 0.08 Mg N ha^{-1}. Another field study showed that during winter, nitrate leaching from perennial grain cropping was 0.1 and 3.1 kg N ha^{-1} yr^{-1} three and four years after its establishment, respectively, which is significantly lower than that from conventional annual wheat cropping, which was 5.6 kg N ha^{-1} yr^{-1} (Huddell et al. 2023). Perennial grain cropping also created favourable conditions for a more complex and diverse soil food web than annual crops (Sprunger et al. 2019; Förster et al. 2024). With these and other known ecosystem services (Figure 1) provided, perennial grain cropping systems have been proposed as a more sustainable alternative to their annual counterparts (Chapman et al. 2022) to reduce the environmental issues associated with annual crops (Soto-Gómez & Pérez-Rodríguez 2022). Thus, perennial grain crops have been suggested as a useful strategy for adapting agriculture to a changing climate (Glover et al. 2010; Jungers et al. 2023).

1.4.2 Opportunities and challenges of the first perennial grain crop

A promising example is the first developed perennial grain crop, trademarked as Kernza®, which has been domesticated from a cool-season perennial forage grass called intermediate wheatgrass (IWG) (*Thinopyrum intermedium* (Host) Buckworth & Dewey) through a breeding programme at the Land Institute in Kansas, USA (Culman et al. 2013). IWG originated from the area between the Black Sea and Caspian Sea in the Stavropol region of Russia, and the area from Kazakhstan to Turkey (Crain et al. 2024). Over the past decade, IWG has been tested in different climates and soil conditions to determine the viability and scope of this new perennial crop and its appropriate agronomic management (Soto-Gómez & Pérez-Rodríguez 2022). IWG has shown the ability to produce more root biomass than annual wheat (Duchene et al. 2020) increase soil C content (Culman et al. 2013) and C stabilisation in deep soil layers (Peixoto et al. 2022), enhance water uptake and maintain water use efficiency throughout the growing season (Culman et al. 2013; Vico & Brunsell 2018; de Oliveira et al. 2020; Clément et al. 2022), mainly due to the long-term vegetative cover of the soil and extensive root systems (DeHaan et al. 2023).

However, at the current stage of development, the grain yield of IWG is 112-1212 kg ha^{-1} (Culman et al. 2013; Law et al. 2022), which is significantly lower than the global average grain yield of conventional annual wheat, 4020 kg ha^{-1} (Dadrasi et al. 2023). Therefore, replacing annual wheat with IWG at this stage would result in serious loss in food production. Successful adoption of perennial crops will depend on improved breeding strategies, digital technologies for root traits selection, and sustainable market modifications (Jungers et al. 2023). Despite the shortcomings regarding yield, the positive effect of IWG on soil C and water retention can potentially affect soil-microbial interaction and soil health. Most of the current studies on IWG have focused on the environmental and economic benefits. A small number of initial studies have investigated the impact of IWG on soil health (Audu et al. 2022; Rakkar et al. 2023).

1.5 Soil microbial organisms play crucial roles for soil health

1.5.1 Soil health and soil quality

Soil health is the capacity of soil to function as a vital living system, within ecosystem and land-use boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and promote plant and animal health (Doran & Zeiss 2000; Bünemann et al. 2018). Soil quality is typically defined as the capacity of a soil to perform its function necessary for its intended end use (Karlen et al. 2003; Bünemann et al. 2018). The use of the term “soil quality” will generally be associated with a soils fitness for a specific use (Doran & Zeiss 2000). Soil health places a great emphasis on soil biodiversity and ecological functions that make soil a dynamic living resource with the capacity for self-organisation (Allen et al. 2011). The distinction between soil quality and soil health has evolved from a matter of principle to a matter of preference (Bünemann et al. 2018). This thesis uses the terms soil health and soil quality interchangeably. In an agricultural context, soil health is defined as the continued capacity of soil to function as a vital living ecosystem that sustains plants, animals and humans (Lehmann et al. 2020) and often described using a set of quantifiable soil physical, chemical and biological qualities called soil health indicators (Wander et al. 2019; Moebius-Clune et al. 2016). It is a proven fact that healthy soils lead to overall improved agronomic yield (Oldfield et al. 2019), enhanced yield stability under extreme weather conditions (Mahmood et al. 2023), and act as a terrestrial C sink (Bossio et al. 2020). The delivery of ecosystem services (Figure 1) for human benefit depends on a healthy and living soil ecosystem.

1.5.2 Soil microorganism groups and their function

Soil fungi and bacteria account for the majority of soil microbial biomass and diversity (Bardgett & van der Putten 2014; Fierer 2017), and they are essential for maintaining or enhancing soil health due to their critical roles in soil aggregate formation, organic matter decomposition and nutrient cycling (Sahu et al. 2017). Soil fungi is a large and diverse group of microorganisms that have different functions and respond differently to environmental changes. Soil saprotrophic fungi decompose soil organic matter and influence nutrient availability for plants (Ning et al. 2021). The arbuscular mycorrhizal (AM) fungi can enhance plant growth by improving water and nutrient uptake through extending plants' root absorbing area (Begum et al. 2019). Soil bacteria is also an extremely large and diverse group of microorganisms. Plant-growth-promoting rhizobacteria (PGPR) promote plant growth, control plant pests, and induce resistance to various abiotic stresses (Saeed et al. 2021). N_2-fixing microbes convert atmospheric N_2 into ammonia (a bioavailable N form) (Bohlool et al. 1992). Gram-negative bacteria are responsible for decomposing the fresh plant-derived C, whereas gram-positive bacteria are more capable of using recalcitrant compounds (Kramer & Gleixner 2006; Tavi et al. 2013). These soil microorganisms produce extracellular polymers, biofilms and hyphae (fungi) that bind root debris, organic matter and soil particles (Costa et al. 2018) that stabilized the soil organic C. Their mineralization activities influence the balance between soil C and atmospheric C differently because fungi and bacteria use different organic and inorganic forms of C from soil and plants root exudates and dead material as energy sources (Gougoulias et al. 2014). Fungi usually efficiently use more recalcitrant C (with high C: N ratio) while bacteria use more easily degradable C (with low C: N ratio) (Hunt et al. 1987; de Vries et al. 2011).

Under drought conditions, soil microbes are crucial for crop adaptation to drought stress through various mechanisms, including improved root volume, nutrient uptake, the content of plant hormones, antioxidant enzyme activity, and relevant gene expression (Gu et al. 2024). For example, AM fungi can increase biosynthesis of metabolites such as proline and sugars to provide osmotic adjustment in plant roots and leaves under drought stress (Behrooz et al. 2019), allowing plants to maintain photosynthetic efficiency and biomass production (Wahab et al. 2023). Plant growth-promoting bacteria can solubilize phosphate and produce phytohormone to improve growth and nutrient acquisition of plants under drought stress (Singh et al. 2024). However, the relationship between soil microbes and plants under drought conditions is complex. A pot study by Ulrich et al. (2019) has shown that inoculation with soil microbes with distinct bacterial and fungal community composition can initially enhance the growth and photosynthesis of a C4 perennial grass, but negatively influence the plant performance during severe drought over time.

The soil biological properties (e.g. microbial biomass, respiration, metabolic substances, community analyses, enzymatic activity), respond more rapidly than chemical and physical properties to environmental changes or changes in agricultural management practices (Doran 1996; Bhowmik et al. 2019; Kumar et al. 2023). This makes them ideal for use as early indicators for soil health changes. Among various bio-indicators the microbial biomass is considered as the most sensitive and effective bio-indicator because it is directly influenced by biotic and abiotic factors (Karlen et al. 2019; Nunes et al. 2020). Soil microbial biomass from soil samples can be estimated by isolation of EL-FAME (ester-linked fatty acid methyl ester) and PLFAs (phospholipid fatty acids) or by qPCR (real-time quantitative polymerase chain reaction) (Frostegård & Bååth 1996; Wallander et al. 2013; Mercado-Blanco et al. 2018). The PLFAs from living cell membranes vary in C chain length, saturation and branching in different microorganisms. This make them ideal signature molecules for identifying different microbial groups and analysing microbial community structure (Willers et al. 2015). They are also useful for determining biomass changes related to soil disturbances such as cropping practices (Hill et al. 2000). The PLFA method was selected for the current studies in this thesis because PLFAs degrade rapidly after cell death and therefore might reflect living microbial biomass. Furthermore, the PLFA method is more sensitive in detecting shifts in the microbial community composition compared to DNA/RNA-based methods (e.g. qPCR). However, it should be noted that the PLFA method cannot provide detailed species composition or phylogenetic resolution when used on its own (Ramsey et al. 2006; Willers et al. 2015). While DNA/RNA-based methods can identify soil microbes at the species level, they cannot provide absolute abundance of taxonomic genes (Fierer et al. 2021).

1.5.3 Soil microbial community structure changes under drought

Soil microbial community composition is sensitive to dry and rewetting cycles. Drought and subsequent rewetting stress can significantly affect soil moisture, soil water potential and oxygen diffusion, and thus the growth and survive of microorganism groups and community structure (Amarasinghe et al. 2023), which impairs their ability to perform crucial ecosystem functions involved in biogeochemical cycling. Fungi and bacteria have different capacities to withstand and recover from disturbance of drought and rewetting due to unique features such as osmolyte production, dormancy, dispersal abilities, and growth strategies (Philippot et al. 2021). From perspective of the *r*- and *K*- selection theory (Andrews & Harris 1986), fungi are typically considered as oligotrophic *K*-strategists and characterised as slow-growing, more stable population size, with great resource investment in defensive structures (Kaiser et al. 2014). Fungi usually demonstrate greater resistance to drought than bacteria (Barnard et al. 2013). However, the fungal community structure can be more affected by rewetting and drying-rewetting cycles than bacteria (Liu et al. 2019). Bacteria are considered as copiotrophic *r*-strategists and characterised as fast-growing under high nutrient availability, with highly variable population size (Fierer et al. 2007). Bacteria usually return to the pre-drought microbial composition upon rewetting, and are more resilient than fungi (Barnard et al. 2013). Gram-positive bacteria have a thick and interlinked peptidoglycan cell wall which can act as protection towards water stress, while gram-negative bacteria have a single-layer cell wall and an outer membrane, making them generally more susceptible to water stress (Schimel et al. 2007). Some gram-positive bacteria have the ability to form spores, which have the capability to take on a dormant cellular form and thus endure extreme conditions (e.g. drought) of their habitat (Andryukov et al. 2021). Fungi, bacteria and other microorganisms are part of the soil food web, and their interaction is undoubtedly influenced by drought and rewetting stress. Research shows fungi can help bacteria cope with drought by forming mycelia and transferring water and nutrients, which stimulates the germination and subsequent growth of bacterial spores near the hyphae in dry and nutrient-free microhabitats (Worrich et al. 2017).

1.5.4 Microbial drought resistance and resilience

The capacities of soil microorganisms to withstand and recover form abiotic stress largely determines the capacity of the soil as a whole to recovery and function after stress. The ability of a system (e.g. soil microbial community) to withstand disturbance is defined as resistance, and the rate at which a system (e.g. soil microbial community) returns to its original state is defined as (engineering) resilience (Griffiths & Philippot 2013). The disturbance required to move the system from one stable state to another different stable state is defined as ecological resilience (Philippot et al. 2021). The engineering resilience is commonly used in microbial ecology. The studies in this thesis use engineering resilience concepts that is the rate of recovery after rewetting drought-treated soils.

It is unclear whether soil function recovers after rewetting, despite the potential for biomass or community structure to do so. It is possible that microbial community composition recovers, but function does not. This is known as physiological adaptation (Philippot et al. 2021). Alternatively, function may recover, but not the community composition probably due to functional redundancy (i.e. different species can perform the same functional role in ecosystems under temporally and spatially varying conditions) (Walker 1992; Philippot et al. 2021). It is therefore critical to study the resilience of soil microbial function to drought disturbance. The resilience of soil microbial biomass, community composition and activity can significantly affect soil nutrient cycling, C sequestration, long-term soil fertility and health. This makes them important indicators for estimating the resilience of soil functions to drought. The resistance and resilience of soil microbial communities are influenced by plant species composition, root exudates, plant dead material, soil physico-chemical structure, substrate quality, and soil management (Griffiths & Philippot 2013). Understand how soil microorganisms and community structure withstand and recover from drought and rewetting stress, and how different crop species and soil management influence the soil microbial resistance and resilience to drought is important for agricultural crop production to enhance soil health and functional resilience to drought under changing climate.

1.6 How does perennial cropping and drought influence soil microbial community?

Perennial grains have a longer lifetime than annual crops, which means they interact with the soil microbiome for a longer period of time. We do not yet fully understand how the soil microbial community responds to the increased crop longevity (i.e. perenniality), undisturbed upper soil layers and sustained fibrous root growth. Recent studies have provided valuable insights into how IWG and annual wheat differ regarding soil microbial community composition (Sprunger et al. 2019; Duchene et al. 2020; McKenna et al. 2020a), microbial biomass (Audu et al. 2022; Rakkar et al. 2023; Taylor et al. 2024) and the related soil C storage capacity (Sprunger et al. 2019; Audu et al. 2022; Taylor et al. 2023), but they have not reached a consistent conclusion regarding which is the dominating microbial community and the magnitude of the response of soil microbial communities to the crop pereniality (i.e. longevity) and impacts on soil health improvements. Furthermore, the extent to which soil microbial communities and soil health indicators are enhanced by perennial grain crops depends on the specific soil type and local climate (Rakkar et al. 2023). Given the growing interest in perennial grain agriculture globally, it is important to evaluate how perennial grain crop production affects soil microbial biomass, communities and soil parameters. Because soil microbial organisms play a vital role in crop and soil health, understanding knowledge about belowground soil-microbe-roots interactions is central for understanding the mechanisms of ecosystem services (Figure 1) provided by perennial grain crops, and their growth and performance under a changing climate.

As mentioned before, drought has a significant impact on soil microbial diversity, community composition, and network stability (Peng et al. 2024). Perennial crops have more extensive and deeper root systems than annual crops. This allows them to access water and nutrients from deeper soil layers even during dry periods (Clément et al. 2022). This may help perennial crops maintain their productivity and soil microbial communities under drought events. To date, there has been no documentation of the response of soil microbial community to drought and rewetting in perennial IWG cropping systems. Previous research has found that in a pot study, inoculation with native AM fungi benefited a perennial oilseed crop (*Silphium*) under dry conditions, and alfalfa under wet conditions, but not the perennial IWG under either dry or wet conditions (McKenna et al. 2020b). In a field study, Emery et al. (2022) showed that drought increases the species richness of AM fungi in a perennial bioenergy switchgrass monoculture when N fertiliser was applied. It is currently unclear how soil microbes and perennial crops response to drought and subsequent rewetting stress and how they interact with and help each other to cope with these abiotic stress.

The intercropping of annual legumes and cereals has been proven to produce more stable fungal networks under drought conditions than monocultures (Peng et al. 2024). Intercropping perennial cereals and perennial legumes establish long-term root facilitative interactions, which may result in even more efficient water and nutrient use as than annual intercropping under drought conditions. Research has evaluated the positive effects of intercropping IWG with perennial legumes on grain yield (Tautges et al. 2018), biomass (Reilly et al. 2022), forage quality (Pinto et al. 2022), N use (Crews et al. 2022), soil C allocation and stabilisation (Peixoto et al. 2022) and deep soil water use (Clément et al. 2022). However, there is a clear gap in our knowledge regarding how IWG and legume intercropping affects soil microbes under drought conditions. It is clear that more research is required to gain a deeper understanding of how cereal-legume-microbe interactions respond to drought stress, particularly in perennial cropping systems.

Understanding and harnessing the potential of soil microbial communities will enable the development of resilient and sustainable agricultural practices and systems that can enhance soil health and contribute to food security in the context of climate change (Mattoo & Gowda 2022). As agricultural crop production in temperate climates is already influenced by, and predicted to become even more affected by summer droughts and altered precipitation patterns due to climate change, there is an urgent need to assess how perennial crops, soil microbes, and their interactions are affected by drought and rewetting stress, in order to develop strategies to optimise the agricultural cropping systems and to adapt to and mitigate climate change.

1.7 Objectives

This thesis aimed to better understand how IWG sole crop and IWG-legumes intercropping influence soil microbial biomass and communities, and how these sole crops and intercrops and their soil microbes respond to drought and rewetting stress compared to annual cereal sole crops.

The specific objectives of the thesis were to:

- determine the intercropping advantages and interactions between perennial IWG and legume companion crops in pots without drought (Paper I) and in the field under drought (Paper II);
- investigate how intercropping advantages in IWG and legume intercropping are influenced by different levels of species relative frequency and N fertiliser (Paper I);
- investigate the effect of crop perenniality on soil microbial biomass, activity and community composition (Paper III);
- explore the interaction between soil microbial communities and IWG-alfalfa intercropping under drought and rewetting stress (Paper IV).

2. Methods

2.1 Research Theory and Framework

A conceptual ecosystem framework (Figure 1), adapted from Wittwer et al. (2021), Barrios (2007) and (Bach et al. 2020) was used in this thesis to link soil and microbial metrics, soil process, function proxies, soil-based ecosystem services, final agroecosystem goods and, indirectly, contributions to the SDGs.

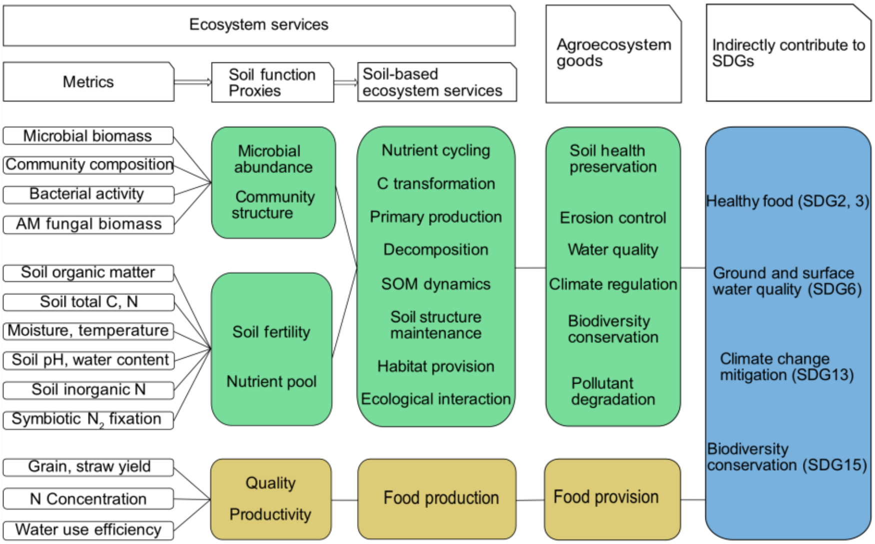


Figure 1. A conceptual ecosystem framework to link soil microorganisms to ecosystem services. The green colour indicates the ecosystem services category Supporting and the yellow colour indicates Provisioning. The blue colour indicates the global goals to which this research indirectly contributes.

This thesis presents a series of scientific studies that emphasise different aspects of the ecosystem services (supporting and provisioning) provided by soil microorganisms and cropping systems. For example, Paper I and II focused on food provision of perennial grain crops, Paper III addressed soil health preservation, and Paper IV highlighted the climate change adaptation and mitigation potential.

2.2 Research Methods

2.2.1 Experimental design in the pot and field studies

The pot experiment in Paper I was conducted in a greenhouse at China Agricultural University in Beijing, China, using a two-factor complete randomised design (Figure 2). The first factor was the application of 4 levels of inorganic N fertiliser: 0, 75, 150, and 225 kg N ha^{-1}. The second factor was the species-relative frequency which had five levels.

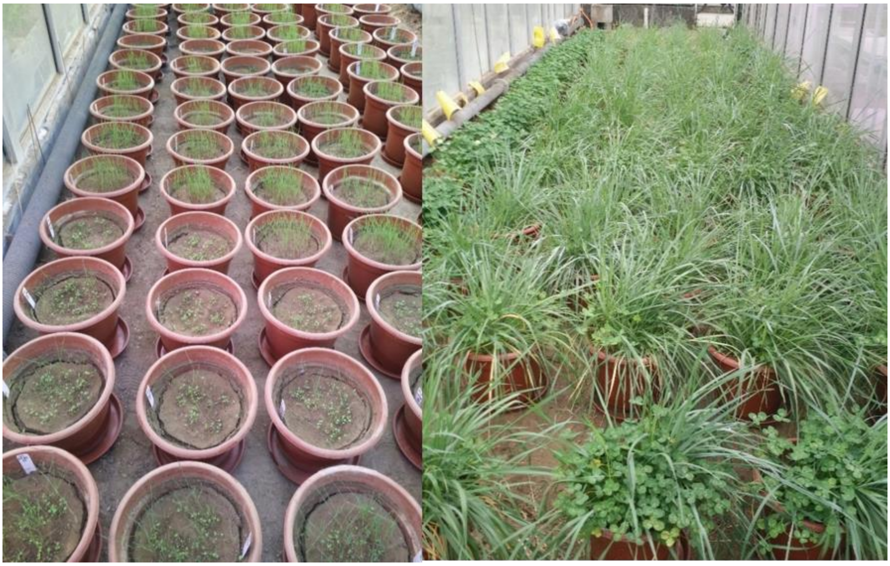


Figure 2. Intermediate wheatgrass and white clover mixed intercropping in pots in a greenhouse. Photograph by Shoujiao Li.

A total of 16 plants were planted per pot in a replacement series design. IWG and white clover (*Trifolium repens* L.) were grown in five mixtures as 100%

IWG (all 16 plants IWG), 75% IWG (12 plants IWG, 4 plants white clover), 50% IWG (8 plants IWG, 8 plants white clover), 25% IWG (4 plants IWG, 12 plants white clover), and 0% IWG (0 plants IWG, 16 plants white clover). Each treatment combination was replicated three times, obtaining a final number of 60 pots of plants. To quantify N in plant biomass as originating from different sources (air, soil, fertilizer), the ^{15}N-labeled ammonium nitrate was used as the N fertilizer to enrich the ^{15}N abundance in soil. For details of the ^{15}N isotopic labelling treatment and agronomic practices, please refer to Paper I.

The field experiments in Paper II, III, and IV all were conducted at the Swedish Infrastructure for Ecosystem Science's (SITES) Agroecological Field Experiment (SAFE) field located at the SITES Lönnstorp Research Station, SLU, in Alnarp (55.65° N, 13.06° E). This region has a humid continental climate with a mean annual precipitation of 533 mm (2015 to 2022) and a daily average air temperature of 10 ºC (Figure 3). The daily air temperature and precipitation data were obtained from the automatic weather station at the research station.

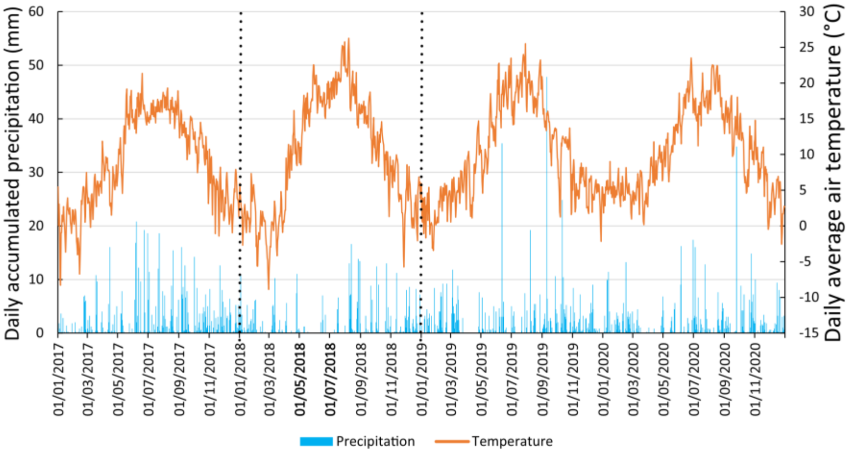


Figure 3. The daily accumulated precipitation and daily average air temperature in SITES Lönnstorp Research Station in 2017 to 2020. The colour blue indicates precipitation and yellow indicates temperature. Dosed vertical lines mark the precipitation in 2018.

The SAFE Field Experiment was established in 2016 and is replicated in four fully randomised blocks with four cropping systems represented in every block. The four cropping systems are: (1) a perennial cropping system consisting of an IWG sole crop and an IWG-alfalfa (*Medicago sativa* L.) intercrop (Figure 4 a), (2) an organic rotation system comprising eight annual crops, (3) a conventional rotation of four annual crops, and (4) an agroforestry cropping system. The soil type was a sandy loam soil, comprising 67% sand and 18% clay. The basic soil physical and chemical properties are reported in Paper II. For a detailed description of the SAFE design, crop planting and field management activities, please refer to the report by Barreiro and Albertsson (2022).

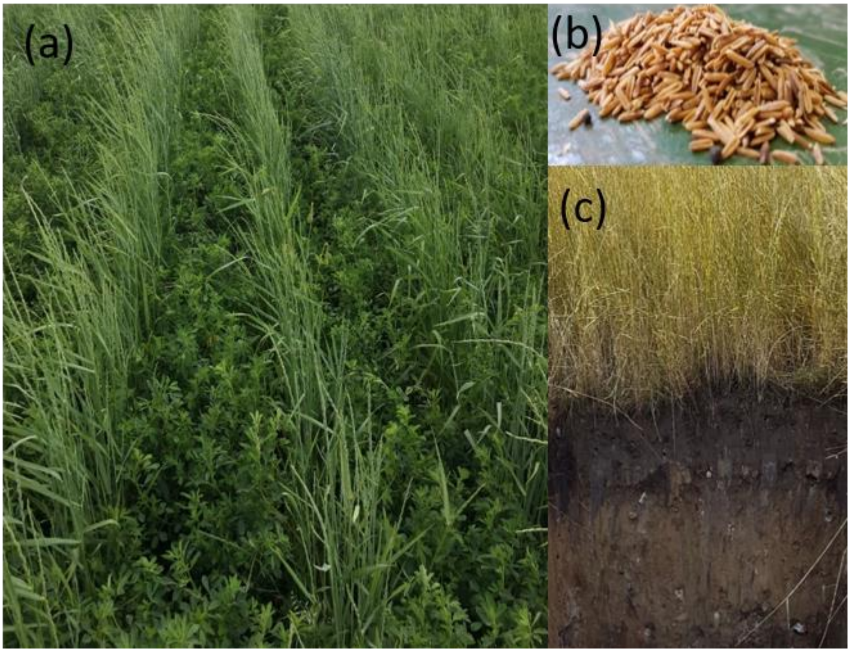


Figure 4. Intermediate wheatgrass (IWG) growing in SAFE field at the SITES Lönnstorp. Left (a) is IWG-alfalfa row intercropping in SAFE field in 2016. Top right (b) is IWG grains after harvest and threshing. Bottom right (c) is soil under the IWG sole crop that have been growing in a seed production plot for 6 years. Photograph courtesies of Erik Steen Jensen, Ryan Davidson, and Ana Barreiro, respectively.

In this thesis, I selected six crops from three SAFE cropping systems based on their perenniality level (i.e. longevity) and management to study the effect of perenniality (Paper III) and drought (Paper IV) on soil microbial biomass and community composition. These six crops are organic IWG sole crop (perenniality level five), organic IWG-alfalfa intercropping (perenniality level five), rye (*Secale cereale* L.) (perenniality level one) or wheat (*Triticum aestivum* L.) (perenniality level one) from the organic rotation, and wheat (perenniality level one) from the conventional rotation. To create three levels of crop perenniality, a biennial grass and legume mixture ley (perenniality level two) from the organic rotation was also sampled and analysed for Paper III. In Paper II, IWG and IWG-alfalfa were the only crops sampled and compared. All experiments in this thesis used the same species and cultivar of intermediate wheatgrass *Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey. The seeds of IWG were obtained from the cycle 3 (2014) germplasm of the perennial grain breeding programme of The Land Institute of Salinas, Kansas, USA. The field management for these six crops was described in detail in supplementary material in Paper III.

Drought was simulated by equipping these five crop plots with rain-out shelters (Figure 5 a-d) to mimic the summer drought that occurred in the region in 2018 (Figure 3).

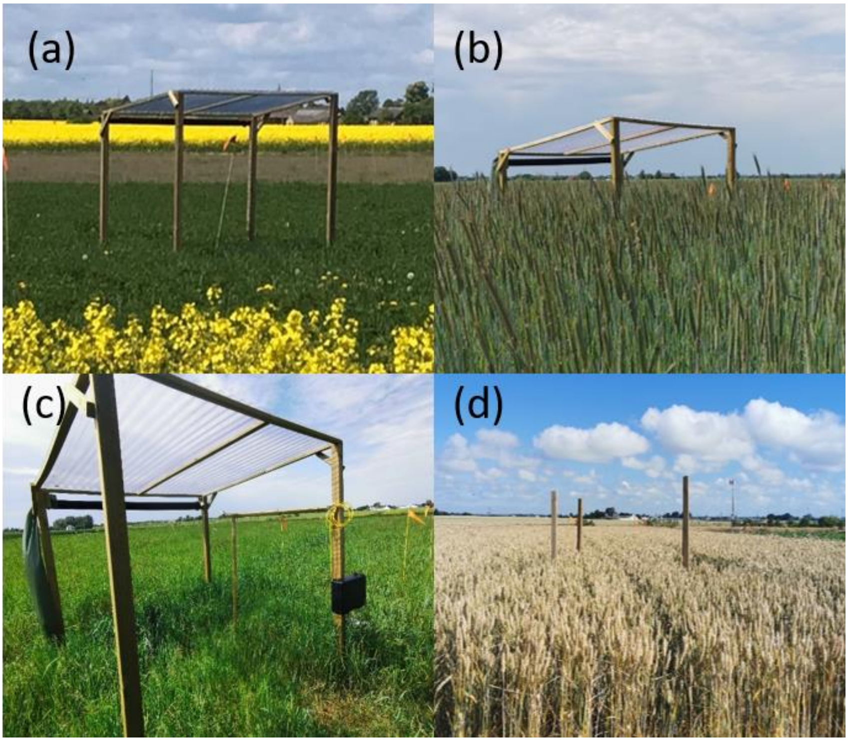


Figure 5. The rain-out shelters in SAFE fields to reduce rainfall and simulate drought. The rain-out shelter in the wheat field in May (a) and July (b). (c) The intercepted rainfall

was collected and led away from the plot using plastic tubing. (d) The rain-out shelters were removed to terminate rainfall reduction. Photography by Ana Barreiro, Linda-Maria Dimitrova Mårtensson and Shoujiao Li.

One shelter (2 m x 2 m) per experimental plot per block was built (n=4). The rain out shelters excluded rainfall from May 17 to July 19 in 2021 and from May 16 to July 11 in 2022, which accounted for 14% of the total precipitation in 2021 (75 mm relative to 538.2 mm) and 16% in 2022 (88 mm relative to 535.4 mm) respectively (Figure 6). The rain out shelters were built in different locations within the plots in 2022 to avoid any additive drought effect. Each rainfall reduction treatment was paired with a natural rain fed control treatment without rain out shelters. The data from the control treatment were used to analyse the effect of crop perenniality on soil microbes in Paper III. The data from the drought and control were used to analyse the effect of drought on soil microbes in Paper IV.

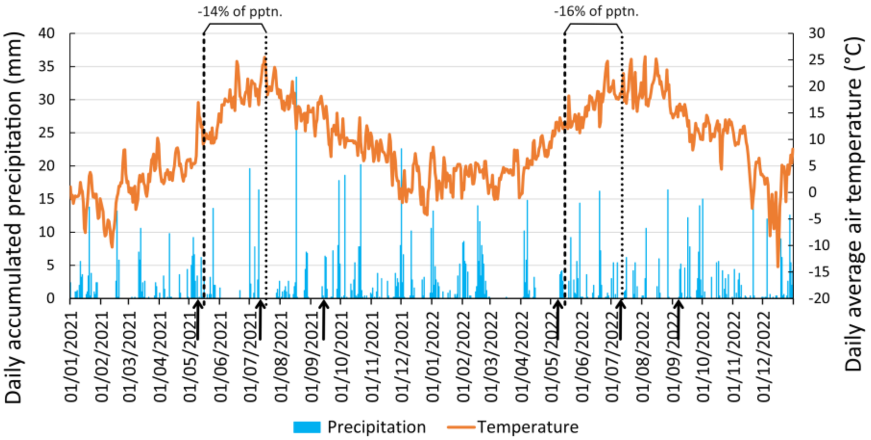


Figure 6. The daily accumulated precipitation and average daily air temperature from January 2021 to December 2022. Dashed vertical lines indicate the dates for rain-out shelter installation (in May) and termination (in July). Arrows indicate the sampling dates. The pptn. indicates precipitation.

2.2.2 Plant and soil sampling

In Paper I, the shoots and roots of IWG and white clover were harvested and separated in July 2019 (the heading stage of IWG). The soil from the pots was sieved (2 mm), homogenised and stored for analysis of inorganic N, water content and pH.

In Paper II, the plant material in IWG and IWG-alfalfa was sampled in four 0.25 m^2 subplots in each experimental plot before harvest in September 2017, August 2018, and September 2019.

In Papers III and IV, the plant samples were taken in one 0.25 m^2 square in the rain-reduced area that 0.25 m away from the rain out shelter edge area, in four cropping systems, in May (7 days before drought), July (after 56 days of drought), and September (57 days after the drought was terminated) in 2021 and 2022 (Figure 6). After plant sampling, the grain (Figure 4-b), straw yield and root biomass were measured as dry matter. This was done by drying the plant material in an oven at 60°C (Paper I, III, IV) or 65°C (Paper II) until a constant weight.

Soil samples were collected (Figure 7) after sampling the plants in the same rain-reduced area. The control samples were collected in the rain fed area in the same plot, 2 m from the rain reduction area. For each plot, four replicate soil cores (2.5 cm diameter) were collected from a depth of 0-40 cm at the corners of each plot, and divided as follows: 0-5, 5-15, 15-30, 30-40 cm. The four soil cores were pooled for each depth before analysis, resulting in a total of 768 soil samples. The soil samples were transported to the laboratory and carefully sieved (2 mm) and homogenised. Subsamples were stored for microbial biomass and community analysis, bacterial growth rate and soil water content, pH, and total C and N analyses.

Figure 7. The soil samples from rainfall reduction area in July. (a) Soils in intermediate wheatgrass (IWG) field; and (b) conventional wheat field. Photography by Shoujiao Li.

2.2.3 Soil microbial biomass and community analyses

The PLFA method was used in Paper III and IV to quantify the changes in living soil microbial biomass and microbial community composition responding to crop perenniality and drought. The neutral lipid fatty acid (NLFA) method was also used to quantify the biomass of AM fungi, as AM fungi store energy as neutral lipids, which other dominant fungi and prokaryotes usually do not (Olsson & Lekberg 2022). Both PLFA and NLFA from soil were extracted and analysed using the single-phase extraction of lipids method developed by Bligh and Dyer (1959) and (Frostegård et al. 1993). An internal standard methyl nonadecanoate (19:0) was added to the phospholipid and neutral lipid fractions to correct errors in volume changes due to solvent evaporation. The PLFA and NLFA were analysed by gas chromatograph (GC) (polar column) with a flame ionisation detector (GC-17A, Shimadzu). For a detailed description of the extraction and analysis process, please refer to Paper III and Paper IV.

To ensure comparability with other studies, the classification of PLFA biomarkers for different microorganisms are listed below: The PLFAs i15:0, a15:0, 15:0, i16:0, 16:1ω9, 16:1ω7t, 17:1ω8, i17:0, a17.0, 17:0, cy17:0, 18:1ω7, and cy19:0 considered to be of bacterial origin (Barreiro et al. 2015); PLFA 18:2ω6 is an indicator for saprotrophic fungi, PLFA 18:1ω9 is a general fungal indicator and PLFA 16:1ω5 indicates AM fungi in the soil; Both PLFA 16:1ω5 and NLFA 16:1ω5 were used as biomarkers for AM fungi (Lehman et al. 2012; Vestberg et al. 2012; Kundel et al. 2020). PLFAs 16:1ω7c, cy17:0, 18:1ω7, cy19:0 were used as indicators for gram-negative bacteria, PLFAs i14:0, i15:0, i16:0, and 10Me18 were indicators for gram-positive bacteria and 10Me16, 10Me17, and 10Me18 were indicators for Actinobacteria.

The abundance of individual PLFA was used for community composition analyses, expressed as relative peak area percentage. The estimated biomass of microorganisms by PLFA concentration was expressed as nanomole (nmole) per gram (g) of organic matter (Frostegård & Bååth 1996) in Paper III and IV. The internal standard 19:0 methyl ester was added to the phospholipid and neutral lipid fractions for fatty acid quantification, but it cannot capture the recovery of the phospholipids from soil after the extraction. Future studies should add a known amount of phospholipid such as 1, 2-dinonadecanoyl-sn-glycero-3-phosphocholine (di19:0 PC) serve as a recovery standard to the soil prior to extraction for quality control (Quideau et al. 2016).

The PLFA method has been used for more than 45 years and it is still popular as a sensitive and rapid tool to indicate community shifts in response to changing environmental conditions (Frostegård et al. 2011). On the other hand, a major shortcoming of this method is its poor taxonomic resolution especially for fungi (Watzinger 2015). The PLFA method cannot provide detailed species composition or phylogenetic resolution when used alone (Ramsey et al. 2006; Willers et al. 2015). When used in combination with other techniques such as stable isotope probing, PLFA can provide insight into the functional component and identify the metabolically active part of the microbial community (Frostegård et al. 2011; Willers et al. 2015). Nevertheless, the PLFA method is useful for providing information on community composition of microbial groups and population dynamics. It has been suggested that functional diversity is usually linked to the community composition of microbes rather than species richness (Roy et al. 2023). PLFA-based estimates of the gram-positive:gram-negative bacterial and fungal:bacterial ratios are usually linked to community growth strategies and can provide general information about microbial community composition shifts and effective nutrient cycling in ecosystems (Wardle et al. 2004; Six et al. 2006; de Vries & Bardgett 2012).

2.2.4 Bacterial growth analysis

The bacterial growth rate was indicated by protein synthesis rate (picomole per g soil per h) and estimated by the radiolabelled leucine incorporation method (Bååth 1994; Söderberg & Bååth 1998; Bååth et al. 2001) in Paper III and IV.

2.2.5 Legume N_2 fixation

The ^{15}N stable isotope dilution method (Jørgensen et al. 1999; Thilakarathna et al. 2016) was used in the pot experiment in Paper I to quantify the N fixed by white clover and the apparent N transfer between IWG and white clover. The ^{15}N natural abundance method (Shearer & Kohl 1986; Unkovich et al. 2008) was used in the field experiment in Paper II and IV to analyse the amount of N fixed by alfalfa.

Both the ^{15}N stable isotope dilution and the ^{15}N natural abundance method are based on the comparison of the ^{15}N composition of legumes and non-N_2-

fixing reference plants, where the difference is assumed to be caused by biological N_2 fixation (Peoples et al. 1989; Høgh-Jensen & Schjoerring 1994). The ^{15}N stable isotope dilution method is more accurate for quantifying N turnover in N cycle processes because it uses enriched ^{15}N (Høgh-Jensen & Schjoerring 1994). However, the ^{15}N enriched material is expensive and it is often difficult to label the soil mineral N pool uniformly (Carranca et al. 1999), which limits the use of this method under field conditions. The ^{15}N natural abundance method, on the other hand, is much more widespread and allows frequent sampling in fields at reduced cost, but the accuracy of the method decreases at lower ^{15}N enrichments in soil N and small natural ^{15}N abundance values may not be detectable by isotope ratio mass spectrometry (Carranca et al. 1999). The accuracy of these both isotopic techniques also depends on the selection of a suitable reference crop which is assumed not to derive any N_2 directly from atmospheric fixation.

The plant C and N concentration, and ^{15}N and ^{13}C abundance were analysed using an elemental analyser coupled with isotope ratio mass spectrometry.

2.2.6 Soil physico-chemical properties

The soil pH was analysed using an electrochemical pH meter in soil: water 1:5 solution (Paper I) or soil: $CaCl_2$ solutions 1:5 (Paper III and IV) after shaking and settling (Schofield & Taylor 1955). The soil inorganic N concentration of extracts was analysed using a continuous flow mass spectrometer by the UV-absorbance spectrophotometer method (Li et al. 2016). The soil total C and N concentrations were analysed by a FLASH 2000 Organic Elemental Analyzer. The soil organic matter concentration was determined as soil loss on ignition at 550°C for 3 hours (Hoogsteen et al. 2015) and calculated according to Moebius-Clune et al. (2016). The soil water content was calculated as the percentage of fresh soil weight loss after drying in an oven at 105°C for 24 hours. Soil moisture, soil temperature and solar radiation were monitored using sensors.

Table 1. Aboveground and belowground parameters measured or estimated directly in the four papers of this thesis.

Parameters	Paper I	Paper II	Paper III	Paper IV
Grain yield		x	x	x
Straw yield		x	x	x
Plant shoot biomass	x			

Parameters	**Paper I**	**Paper II**	**Paper III**	**Paper IV**
Plant shoot N	x	x	x	x
Plant shoot ^{15}N	x	x		x
Plant root biomass, N and ^{15}N	x			
Plant shoot ^{13}C		x		x
Soil pH	x		x	x
Soil inorganic N	x			
Soil total N			x	x
Soil total C			x	x
Soil organic matter			x	x
Soil water content			x	x
Soil moisture			x	x
Soil temperature			x	x
Soil microbial biomass			x	x
Soil microbial community composition			x	x
Bacterial growth rate			x	x

2.2.7 Calculations

The partial land equivalent ratio was calculated for IWG according to Willey (1979) and was used in Paper II to indicate the intercropping advantage. A similar indicator, the relative yield total, was used in Paper I to estimate the relative advantage of mixed intercropping compared to sole cropping (Jensen et al. 2020). The competitive ratio was also used in Paper I as an indicator to evaluate the competitive ability of IWG and legume intercrops (Willey & Rao 1980).

The N accumulation in the plant was calculated as the product of N concentration and dry matter. The discrimination against ^{13}C (Δ^{13}C) during photosynthesis was calculated according to Farquhar et al. (1989) and O'Leary (1988) to estimate the water use efficiency in Paper II.

The ratio between NLFA 16:1ω5 and PLFA 16:1ω5 was used to indicate the structure and abundance of AM fungi in the soil (Olsson et al. 1997; Vestberg et al. 2012) in Paper III and Paper IV. The ratio between fungi indicator PLFA abundance and bacterial indicator PLFA abundance, ratio between AM fungal PLFA relative to saprotrophic fungal PLFA, and the ratio between gram negative bacterial and gram positive bacterial PLFAs were used to indicate community composition changes in Paper III. The drought resistance and resilience values in soil microbial biomass were calculated according to (Orwin & Wardle 2004) in Paper IV.

2.2.8 Statistical analysis

The Analysis of Variance (ANOVA) method was used in all papers in this thesis, based on the complete and balanced data sets, to analyse the effect of treatments on plant- and soil-based parameters (Table 1). Generally, two-way ANOVA was used in all papers to analyse the effects of treatments on aboveground plant-related parameters. For example effects of “N level”, and “species relative frequency” in Paper I, “crop” and “year” in Paper II , and “crop” and “soil depth” in Paper III and IV. Three-way ANOVA was used to analyse the effects of “crop”, “soil depth”, “sampling time” and their interactions on belowground soil-related parameters in Paper III, and the effects of “drought”, “crop”, “soil depths” in Paper IV. One-way ANOVA was used in Paper II to analyse the effect of “year” on N_2 fixation in alfalfa, and effect of “crop” on yield and N content changes in Paper IV. The data was transformed accordingly to fulfil the assumptions of normality and homogeneity for ANOVA.

Paper I used a general linear model (GLM) in IBM SPSS Statistics 23.0 to conduct two-way ANOVA and Tukey’s HSD test for multiple comparisons. Paper II used a proc MIXED model in SAS (SAS 9.4) with the Kenward-Rogers method to conduct two-way and one-way ANOVA, Tukey’s post hoc test for multiple comparisons and Pearson correlation in IBM Statistics SPSS software for analysing correlation. In Papers III and IV, the effects of the treatment were fitted in a linear mixed-effects model that were created by the “lme4” R package and analysed by ANOVA using R statistical software (R studio, version 4.2.0). Pairwise comparisons of means were conducted with Tukey’s method adjustment for multiple comparisons. The Kendall correlation method was used for analysing correlations. All the statistical tests in this thesis use $\alpha=0.05$ as the significance level of effects.

In Paper III and IV, multivariate analyses were used to show the patterns of PLFA data and to discern which ones were the explanatory variables. The relative abundance of individual PLFAs were used to coordinate the data for multivariate analyses. In Paper III, the constrained ordination method, Partial Redundancy Analysis (pRDA), was used to explain the variation in the soil

PLFA profile due to environmental variables after accounting for the variation explained by blocks. Stepwise Regression Forward Selection (based on 999 permutations) was conducted in R to select the relevant explanatory variables, and Holm correction was used to correct the significance level. In Paper IV, the non-metric multidimensional scaling (NMDS) analysis with Bray Curtis distance was used to obtain data ordinations for soil microbial PLFAs relative abundance in two dimensions. This allows us to visualize the similarity and dissimilarity between drought and control, rewetting and control, and among different crops. NMDS plots were fitted with relevant and independent environmental variables using the function envfit from the “vegan” package in R studio (Oksanen et al. 2013). Both Paper III and Paper IV used the permutational multivariate analysis of variance method (PERMONVA) to analyse the significance of the differences between treatments. Pairwise Adonis (ADONIS) tests were used for Post hoc analyses and multilevel pairwise comparisons by using the “vegan” package (Oksanen et al. 2013) in R studio.

3. Results

3.1 Dry matter, N use and species interaction in perennial IWG-white clover intercropping under different N fertilisers and relative frequency (Paper I)

The key results of Paper I showed that: (1) In a pot experiment, reducing the relative frequency of IWG in IWG-white clover intercropping from 75% to 25% did not influence the shoot and root dry matter of IWG intercrop compared to IWG 100%, while the dry matter of white clover intercrop increased (Figure 8). (2) The white clover intercrop had a higher rate of N_2 fixation than the white clover sole crop, and excessive inorganic N fertiliser application decreased the proportion of N derived from air. (3) On average, around 10% of N in the IWG intercrop was transferred from the white clover. (4) IWG could absorb N from soil and ^{15}N labelled fertiliser even when there was a high level of inorganic N fertiliser application. (5) IWG-white clover intercropping had complementarily use of different N sources and facilitative interactions.

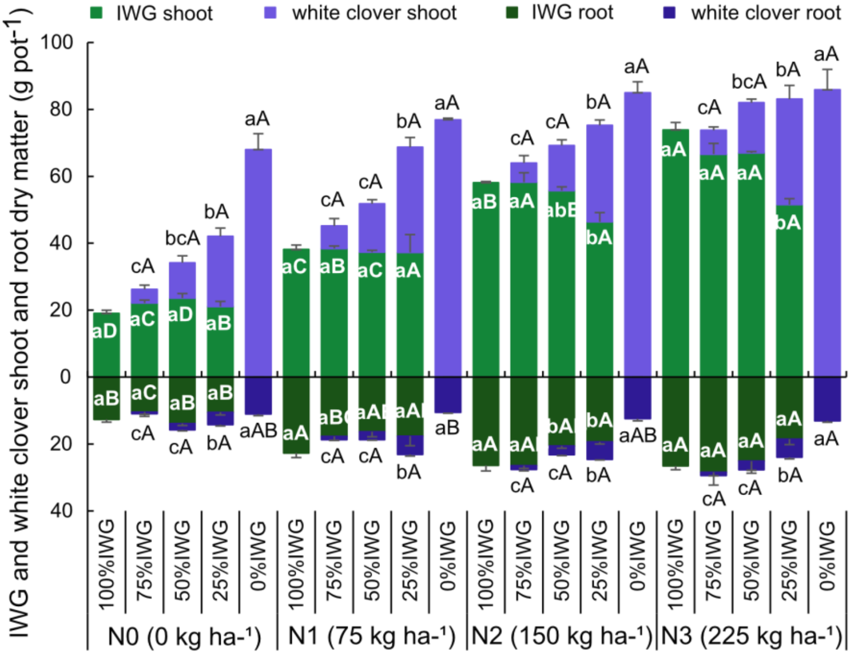


Figure 8. Shoot and root dry matter of intermediate wheatgrass (IWG) and white clover under four N fertiliser levels and five relative frequencies of IWG. Different lower-case letters indicate significant differences among relative frequencies of IWG, and different upper-case letters indicate significant differences among N levels. Light violet indicates white clover shoots, and dark violet indicates white clover roots. Light green indicates IWG shoots and dark green indicates IWG roots.

3.2 Perennial IWG-alfalfa intercropping improved N acquisition, water use and yields sustained under drought (Paper II)

The results of Paper II showed that (1) in field IWG-alfalfa intercropping maintained IWG grain yield and N content under the dry conditions of 2018 and in the following year compared to IWG sole crop (Table 2). (2) The IWG intercrop had higher grain N concentration and water use efficiency (indicated by lower $\Delta^{13}C$) than in the IWG sole crop under the dry conditions of 2018 (Table 2).

Table 2. The grain yield, grain N concentration (%), grain N content and whole plant $\Delta^{13}C$ in intermediate wheatgrass (IWG) sole crops and intercrops in 2017, 2018 and 2019 from Paper II. Data from the dry year 2018 are highlighted in bold text.

Year	**IWG**	**Yield (t ha^{-1})**	**N (%)**	**N content (kg ha^{-1})**	**$\Delta^{13}C$ (‰)**
2017	Sole crop	0.877±0.139a	3.01±0.029ab	16.2±2.29a	20.3±0.20a
	Intercrop	0.553±0.073b	3.45±0.057a	12.1±1.52bc	20.4±0.12a
2018	Sole crop	**0.359±0.026c**	**2.69±0.060b**	**9.62±0.49bcd**	**18.4±0.20c**
	Intercrop	**0.384±0.048c**	**3.39±0.073a**	**13.0±1.59ab**	**17.6±0.33d**
2019	Sole crop	0.261±0.015c	2.66±0.23b	6.96±0.98d	19.0±0.29b
	Intercrop	0.278±0.026c	3.11±0.39ab	8.63±1.32cd	19.1±0.21b

3.3 Effects of crop perenniality on soil microbial biomass, community, activity and soil total C at different soil depths during different seasons (Paper III)

The key results of Paper III were (1) crop perenniality influenced the soil microbial community structure, despite climate factors including precipitation, soil temperature and water content being the main drivers of the variations in the microbial community (Figure 9). (2) The introduction of perennial crops, coupled with reduced tillage and low N input management, led to a notable increase in the proportion of fungi relative to bacteria, AM fungi to saprotrophic fungi, gram-negative bacteria to gram-positive bacteria, and the growth rate of total bacteria. (3) Perennial crops had higher total microbial biomass and microbial biomass of microorganism groups compared to annual wheat (Figure 10), and higher soil total C concentration and C mass in the 0-5 cm soil layer. (4) There was a positive correlation between soil microbial biomass C concentration and soil total C concentration.

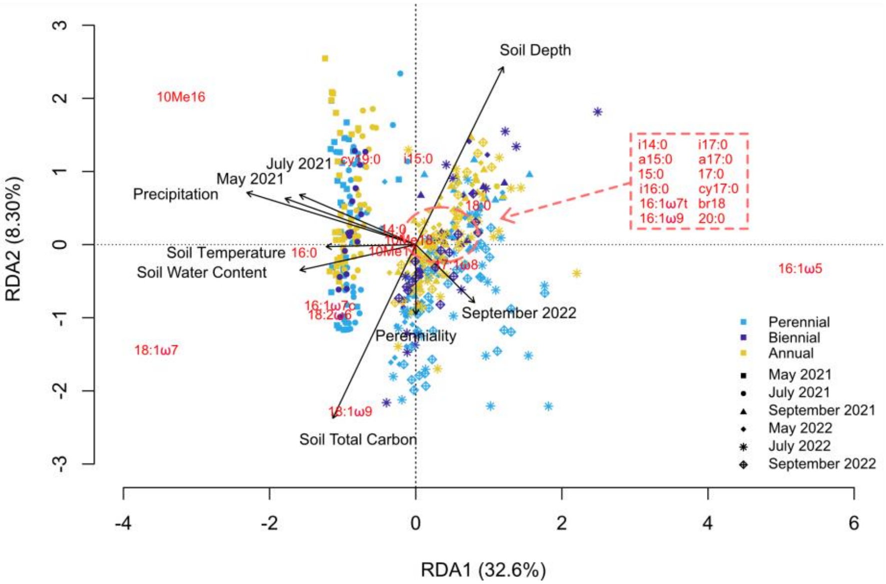


Figure 9. Partial redundancy analysis (pRDA) of soil microbial PLFA profiles in 2021 and 2022 from Paper III. Colours indicate crop perenniality: sky blue indicates perennial crops that were five years old including both IWG sole crop and IWG-alfalfa intercrops, dark blue indicates biennial crops that were two years old including only ley crop, and yellow indicates annual crops that were one year old including organic winter wheat, rye and conventional winter wheat. Symbols indicate sampling time points. Arrows indicate the direction in which the gradient of the environmental variable was greatest, and the length of arrow indicates the strength of the correlation between the environmental variables and the microbes. Only significant variables based on stepwise regression forward selection are displayed. The first constrained axis RDA1 and the second constrained axis RDA2 have the largest contribution to the variance.

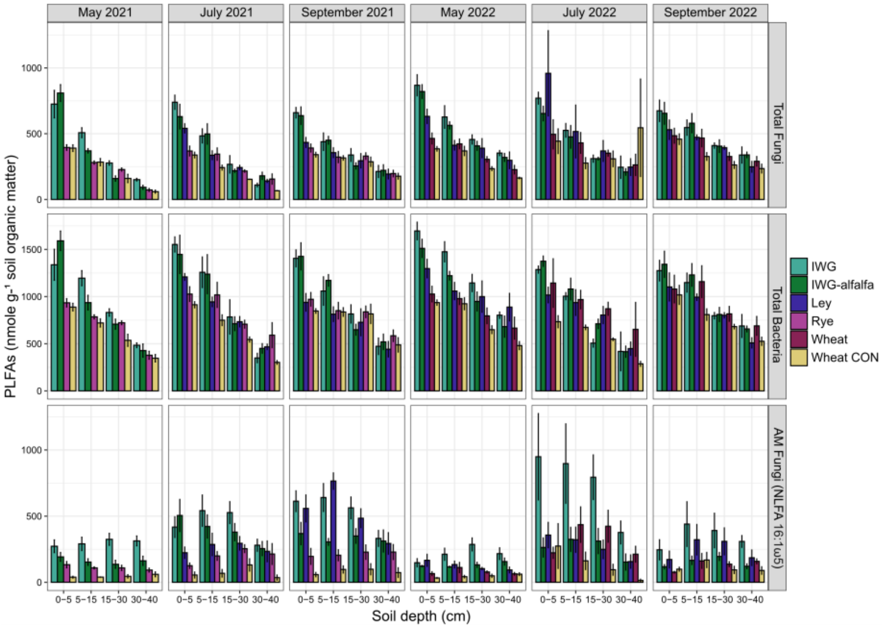


Figure 10. Quantification of soil microbial PLFA profiles at four soil depths in 2021 and 2022. Colours indicate a specific crop: turquoise indicates the perennial intermediate wheatgrass (IWG) sole crop under organic management, green indicates intercropped IWG with alfalfa under organic management, dark blue indicates the biennial ley crop under organic management, pink indicates annual rye under organic management, dark purple indicates annual wheat under organic management, and yellow indicates annual wheat under conventional (CON) management.

3.4 Effects of in situ rainfall reduction on crop productivity, soil microbial biomass and community in perennial and annual cropping (Paper IV)

The results of Paper IV showed that (1) drought generally increased soil microbial biomass, while rewetting reduced microbial biomass (Figure 11). (2) The perennial IWG-alfalfa intercropping had the highest microbial biomass gain and loss under drought and rewetting respectively (Figure 11), and the highest drought resilience. (3) The perennial IWG sole crop had higher drought resistance in soil microbial biomass and less grain yield loss compared to annual crops. (4) The amount of drought resistant AM fungi at 15-30 cm is negatively correlated with the crop grain reduction occurred under drought conditions.

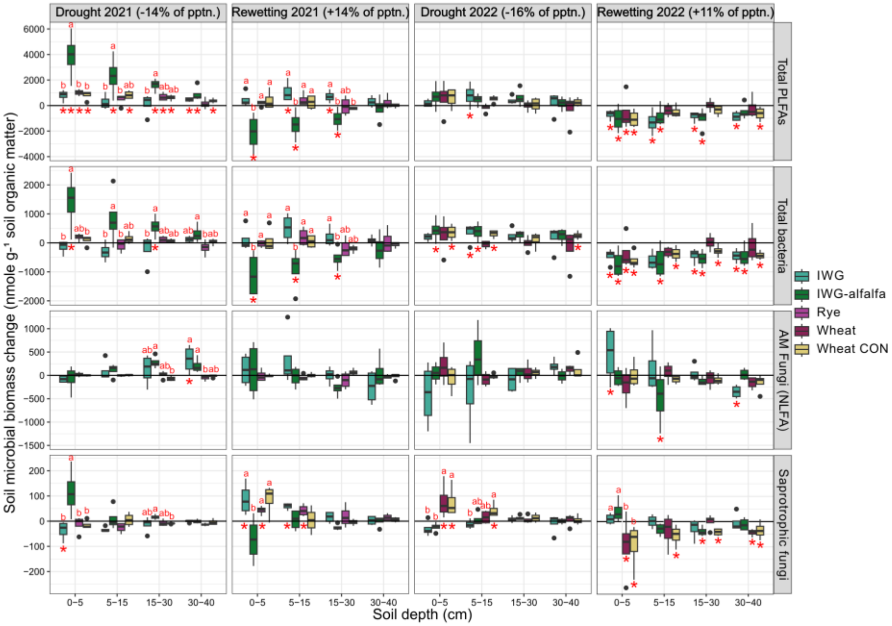
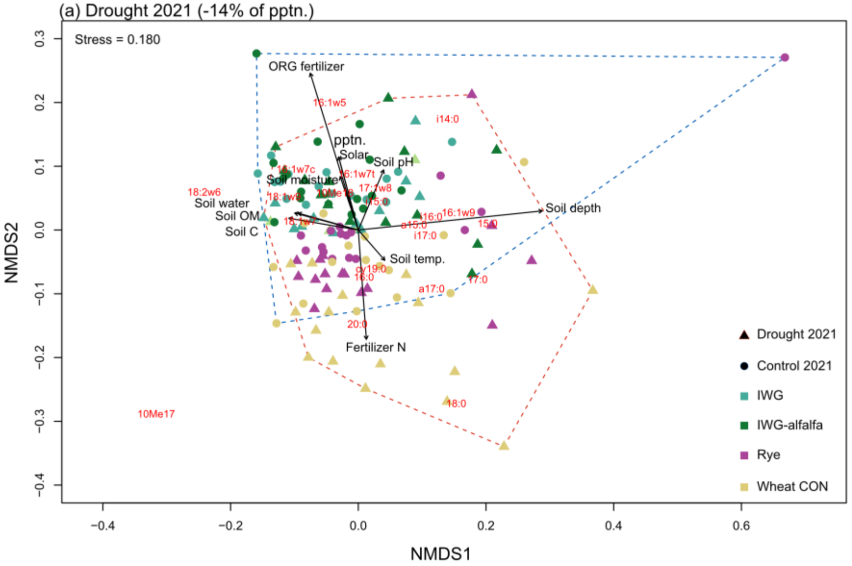


Figure 11. Changes in estimated total soil microbial biomass, total bacterial biomass, AM fungal biomass and saprotrophic fungal biomass due to drought and rewetting at four soil depths in 2021 and 2022 from Paper IV. Values are indicated by the differences between treatments and control. A positive value indicates biomass gain, a negative value indicates biomass loss, and 0 indicates no change by treatments. Statistical significance of treatments is indicated by asterisk, and significance of differences between crops is indicated by compact letters. Colours indicate a specific crop; turquoise indicates organic IWG sole crop, green indicates organic IWG-alfalfa intercrops, pink indicates organic rye, dark purple indicates organic wheat, and yellow indicates conventional (CON) wheat.

The results of Paper IV also showed that (5) drought simplified community composition with less variety of PLFAs, and rewetting shifted community composition completely (Figures 12; 13). (6) The shifts in microbial community composition were mainly driven by changes in soil water content, soil moisture, soil temperature, and soil depth, with the effects of crop species being crucial (Figures 12; 13). (7) Perennial IWG sole cropping and IWG-alfalfa intercropping significantly altered the microbial community compared to annual cereal sole crops, making it more abundant in AM fungi, saprotrophic fungi and general fungi and bacteria under both drought and rewetting (Figures 12; 13).

(a) Drought 2021 (-14% of pptn.)



(b) Rewetting 2021 (+14% of pptn.)

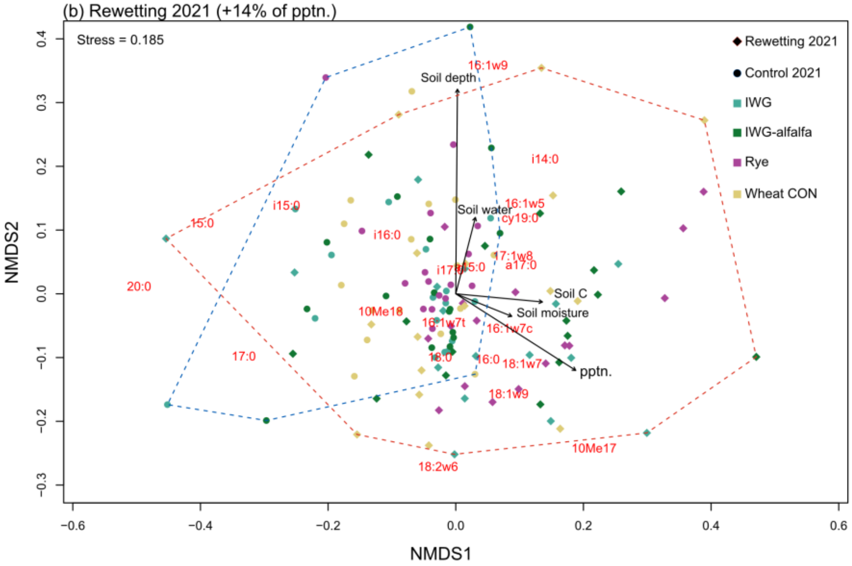
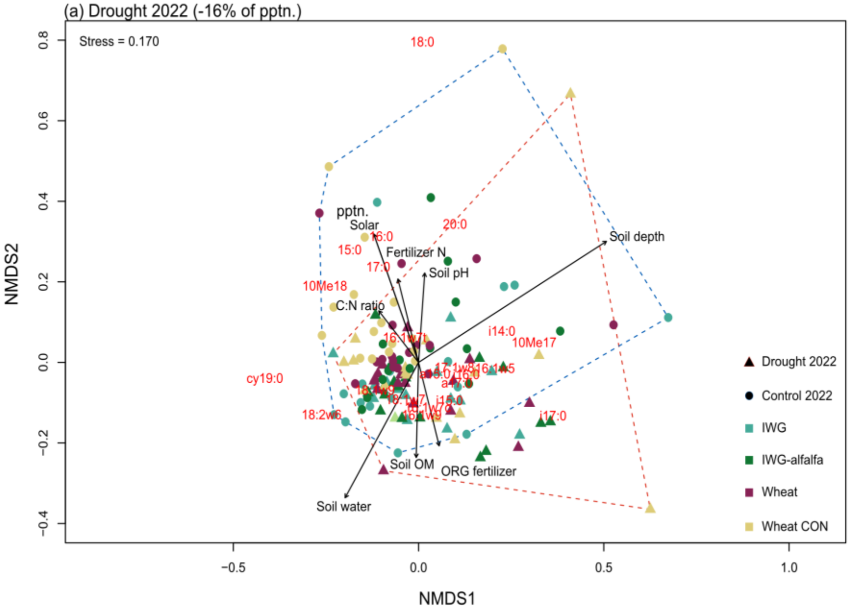


Figure 12. Non-metric multidimensional scaling (NMDS) of soil microbial PLFAs under drought (a) and rewetting (b) in 2021. Convex polytopes were drawn on NMDS plots using sampling points under treatments and control to visualise the difference between them. Colours indicate a specific crop. The triangle indicates in-situ drought simulation, diamond indicates rewetting after drought-treated, and circle indicates rain-fed control.

(a) Drought 2022 (-16% of pptn.)



(b) Rewetting 2022 (+11% of pptn.)

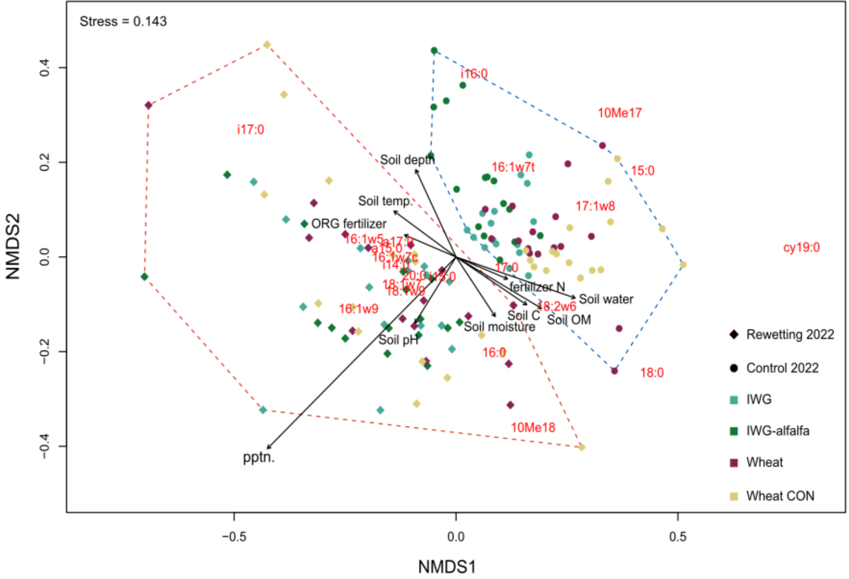


Figure 13. Non-metric multidimensional scaling (NMDS) of soil microbial PLFAs under drought (a) and rewetting (b) in 2022. Convex polytopes were drawn on NMDS plots using sampling points under treatments and control to visualise the difference between them. Colours indicate a specific crop. The tringle indicates in-situ drought simulation, diamond indicates rewetting after drought-treated, and circle indicates rain-fed control.

4. Discussion

4.1 The role of legume companion for improving perennial grain crop yield, N and water use under dry conditions (Paper I and II)

Paper I and II showed that IWG and legumes intercropping had intercropping advantage in terms of dry matter yield and N content in both field and pot experiments. The intercropping advantage was even more obvious under drought conditions in the field experiment, and under low inorganic N fertilizer input in the pot experiment. The current studies demonstrated that drought and low N inputs facilitated the cereal-legume intercropping advantage. This aligns with the results of other intercropping studies (e.g. Chamkhi et al. 2022) which showed that in the situations of limited water resources and/or low-input farming systems, intercropping can improve the growth and performance of both intercropped crop species. The intercropping advantage occurs in these resource-limited systems because cereal and legume intercrops use resources more efficiently than sole crops (Jensen 1996; Hauggaard-Nielsen & Jensen 2001; Rodriguez et al. 2020). This is due to niche differentiation, complementary use of different resources, and potentially apparent N transfer (Bedoussac et al. 2015). Our pot study (Paper I) once again confirmed these mechanisms. The pot experiment demonstrated that under low level of N fertilizer application (N1), white clover relies on the N derived from the air (>80%) through biological N_2 fixation, saving soil N for IWG acquisition (>50%) (niche differentiation). The pot experiment also demonstrated that white clover transfers N to IWG (apparent N transfer). The excessive N fertiliser application impaired the complementary effect and the intercropping advantage between white clover and IWG.

In our field study (Paper II) in dry year 2018, the water use efficiency and N concentration in the IWG intercrop were higher than that in the IWG sole crop indicating that intercropping with alfalfa provided a better N acquisition and water status for the IWG intercrop, enabling it to sustain photosynthesis and growth (Paper II). This is in line with other studies (e.g. Yin et al. 2020) which have demonstrated that water use efficiency is higher in intercropping than in sole crop under stressful conditions such as water and N deficiency. This is probably attributable to decreased evaporation loss through better vegetative cover in intercropping and complementary interactions for water uptake due to differences in root distribution across soil layers (Van Duivenbooden et al. 2000; Layek et al. 2018). This mechanism needs to be studied further in future studies. The belowground root-root interactions were influenced by pot volume and in the pot experiment, where the crop species are mixed and roots were intermingled, which may amplify the root-root interactions (including facilitation, interspecific and intraspecific competition). In the field, where crops are intercropped alternatively in rows, roots are not limited by space, and therefore interspecific competition is likely to be less than in a pot. This allows two crop species to develop complementary root systems allocation. Studying root allocation of intercrops in different soil layers and the impact of distance between two intercrops would provide more information on belowground interactions between IWG and legume and their further contribution to intercropping advantage. Nevertheless, paper I and Paper II, suggest that perennial IWG and legume intercropping is an effective management tool for improving production security in the face of extreme and frequent drought events caused by climate change.

Our results further demonstrated that management strategies such as choice of companion legume species, adjustments in species relative frequency of intercrops and N fertilizer level can significantly influence and increase the advantages of intercropping. Our previous pot study (Li et al. 2020) showed that alfalfa is too aggressive when sown with IWG at the same time. Paper I showed that white clover had better compatibility when mix-intercropped with IWG than alfalfa, in terms of low competition for inorganic N and probably light as well. A recent study by Fagnant et al. (2023) suggests that IWG can reach high aboveground dry matter under low N availability, and the highest aboveground dry matter was found for a 100 to 150 kg N ha^{-1} N fertiliser application over the entire growing year. Our pot study showed that white clover was able to fix 11.8 kg N t^{-1} dry matter, and the field study showed alfalfa can fix 19.0 kg N t^{-1} dry matter. The white clover is capable to fix more N_2 in field (55 kg N t^{-1} dry matter) as showed in a review (Carlsson & Huss-Danell 2003). Our result suggesting that the incorporation of a legume could serve as an effective way to limit the need for N fertiliser inputs for IWG.

4.2 Perennial crops shape the soil microbial community and increase the soil C in the upper soil layer (Paper III)

Paper III focused on the soil microbes under IWG-alfalfa intercropping, IWG solo cropping, and other biannual (ley cropping) and annual sole copping systems. It studied the dynamics of soil microbial biomass and community composition at four soil depths during plant growing seasons in the field. The results showed that crop perenniality (longevity) and no-till management practices shaped soil microbial community structure, despite the climate factors were the main drivers. High levels of perenniality (5 yr) in IWG and IWG-alfalfa lead to a significant increase in the abundance of fungi and AM fungi relative to bacteria and saprotrophic fungi. This is likely a result of long-term (5 yr.) soil cover, root exudate input, the absence of tillage practices (Helgason et al. 2009), and low N fertilizer input in perennial cropping systems, which favour the growth of fungi over bacteria (Wardle et al. 2004; de Vries & Bardgett 2012).

A higher level of perenniality was correlated with high soil total C concentration. The higher soil C concentration in shallow soil layers observed in perennial IWG cropping is in line with the results reported by other studies (Sprunger et al. 2019; Audu et al. 2022). This is attributable to the fungal-based food webs which have been shown to retain greater ecosystem N and C than bacteria-based food webs due to the slower rates of N cycling (Wardle et al. 2004; de Vries et al. 2012) and C turnover (Holland & Coleman 1987; Bailey et al. 2002; Six et al. 2006). Notably, perennial crops harboured higher amounts of living soil microorganisms including bacteria, fungi, AM fungi, saprotrophic fungi, gram-negative, gram-positive and actinobacteria associated with higher soil C concentration at shallow soil layers compared to annual crops. This is probably attributable to the continuous organic substrates input from root exudates and residues, which provide energy and C for soil microbes' biosynthesis (Sun et al. 2016), and a lack of soil disturbance by tillage. As the base of the soil food web, fungal and bacterial biomass and community likely influence other organisms at higher trophic levels like bacterivorous organisms, such as bacteria-feeding nematodes, protists, and fungivorous organisms, such as fungal-feeding nematodes and mites (Adl et al. 2012). Increased biomass of soil bacteria and fungi may support the population of such organisms at higher trophic levels and form a complex soil food web, which can have positive effects on nutrient cycling and soil health, and therefore plant productivity (Mikola & Setälä 1998; Sackett et al. 2010).

Although the link between soil microbial biomass and function remains unclear (Fierer et al. 2021), it is indisputable that microbial biomass and necromass are the primary C-containing constituent contributing to stable soil organic matter (Wu et al. 2024). As shown in literature, after soil microbial death, the mean necromass of soil microbes contributes to 24% 60% of the soil organic C pool (Deng & Liang 2022). Our findings demonstrate a clear and positive correlation between soil microbial biomass C concentration and soil total C concentration which indicate a positive effect of soil microbial biomass and anabolic activity on soil C storage, particularly in the context of perennial cropping. On the other hand, C can be released from soil to the air as CO2 through microbial catabolic activity such as decomposing soil organic matter (Liang et al. 2017). The fate of the increased soil total C mass at the upper soil layer under perennial cropping depends on the balance between microbial catabolic and anabolic activities, which can be influenced by biotic and abiotic factors such as litter input quality, soil C: N ratio, and other climate drivers (Liang et al. 2017). In the perennial IWG cropping systems, reduced tillage activities imply less disturbance on soil microbial community and may facilitate the downward translocation of the soil C into the deeper soil profile as a more stable C form (Nicoloso et al. 2018). Future studies on C fraction, mineralisation rates and root exudates' contribution could provide greater insight into soil C dynamics, which could be better correlated with soil microbial community composition shift and microbial activity.

The intercropping of IWG and alfalfa did not result in significant changes in the estimated biomass of soil microorganism groups and their ratios compared to IWG sole crop in most combinations of sampling time points and soil depths under natural conditions in Paper III. However, under drought stress, the soil microbes in IWG-alfalfa intercropping performed better than the IWG sole crop in terms of resilience in biomass and community composition.

4.3 Microbes in perennial sole and intercropping are more drought resistant and resilient than that in conventional annual cropping (Paper IV)

Paper IV has studied the responses of soil microbial biomass, community and activity from different cropping systems to drought and rewetting in the field, and explored the linkage among diverse, drought-resistant soil microbes and crop agronomic performances. Paper IV has confirmed that the IWG-alfalfa intercropping had greater drought resilience in soil microbial biomass and a more stable community composition than the IWG sole crop. The high drought resilience in the IWG-alfalfa intercropping was demonstrated by the rapid recovery of soil microbial biomass, which was initially increased after drought and then decreased following subsequent rewetting in 2021. This is contrary to what could be expected. It has been suggested that drought may decrease the microbial respiration and biomass due to reduction in moisture, while rewetting may increase the microbial biomass (Zhang et al. 2015; Yu et al. 2021) due to sudden nutrient pulses of released accumulated organic matter after rewetting (i.e. the “Birch effect”) (Birch 1958; Fierer & Schimel 2003). The reason of these unexpected results could have been that the response of microbial biomass to drought depends on the intensity and duration of the drought stress. The rainfall reduced by our treatment was only 14% of the annual precipitation in 2021 and 16% of the annual precipitation in 2022. That reduction was only half of the amount of the rainfall reduced by the dry summer in 2018, which was 29% of the mean annual precipitation from 2015 to 2022 (2018 excluded). As the drought intensity in 2018 in Sweden was severe, then the drought intensity of our treatment was moderate. Therefore, it is likely that microbial biomass may have benefited from moderate drought as seen in our current study, which is consistent with several previous studies (Sanaullah et al. 2011; Schaeffer et al. 2017; Singh et al. 2021). The reasons are as follows: (1) microbes can survive in hydrologically disconnected microsites that formed as soils dry, because small soil patches might retain hydraulic connectivity under dry conditions, even though despite bulk soils haveing negligible diffusivity at the macroscale (Manzoni & Katul 2014); (2) microbes surviving in these microsites may gain protection from predators and/or viruses, and reduced mortality rather than increased activity may explain the increased biomass (Ranjard & Richaume 2001; Parker & Schimel 2011; Homyak et al. 2017); and (3) moderate drought increases rhizodeposition, root exudates and available substrate for microbes, but under extreme drought rhizodeposition is more variable (Preece & Peñuelas 2016). More severe drought events decrease water potential, reduce nutrient availability and substrate diffusion, and can have a negative effect on microbial growth or even lead to cell death (Lamersdorf et al. 1998). The simulated drought in 2022 seems more severe than that in 2021 due to a longer drought period in April 2022 before the rainfall reduction treatment began. The higher drought severity in 2022 compared to 2021 are attributable for the less increase in microbial biomass under drought in 2022. The microbial biomass responses in IWG-alfalfa intercropping was inconsistent over two years indicating the significant impact of drought period length and intensity on microbial responses.

The intercropping of IWG and legume alfalfa had the highest microbial biomass under drought in 2021 probably due to the capacity of alfalfa to utilise deep soil moisture (Humphries & Auricht 2001), and increased root exudation of carbohydrates and N (Karlowsky et al. 2018). Literature shows that drought increases carbohydrates accumulation in alfalfa roots (Echeverria & Gonzalez 2021) and the total organic C amount in crested wheatgrass root exudates (Henry et al. 2007). This might increase the content of soluble sugars in root exudates in intercropping, which, as a non-specific, easily accessible resource, stimulates the growth and biomass of the entire active microbial community (Landi et al. 2006; Karlowsky et al. 2018). Drought has been proven to increase the concentration of metabolites such as abscisic acid, proline, and flavonoids in alfalfa roots (Echeverria & Gonzalez 2021), as well as fumaric and succinic acids in crested wheatgrass root exudates (Henry et al. 2007), which might stimulate specific microbial functional groups and reduce others (Czaban et al. 2018). For example, root exudation of proline can stimulate the movement of symbiotic Sinorhizobium meliloti towards the alfalfa roots to initiate nodulation and symbiotic N fixation (Webb et al. 2014). Alfalfa roots also exude organic N into the soil, which serves as N resources in addition to C for microbial growth, given that the ability of alfalfa to fix N was not influenced by drought in the current study (Paper IV). Further studies are needed to confirm and quantify the contribution of legume root exudates to drought resilience of soil microbes in perennial intercropping.

The IWG sole crop had higher drought resistance in microbial biomass than IWG-alfalfa intercropping and annual crops. This was evident from the smaller changes in microbial biomass in response to drought and rewetting. This implies that the responding microbial species in IWG-alfalfa intercropping were alfalfa-specific, although the species could not be identified by the current PLFA method, the different impacts between IWG and IWG-alfalfa on microbial community composition were statistically significant during drought and rewetting periods. These fast-responding soil microbes in IWG-alfalfa seemed to benefit from N availability from alfalfa roots under drought. We do not yet know if these microbes are root-associated bacteria that alfalfa plants recruit to enhance their adaptation to drought stress (Fan et al. 2023) or if they are free-living microbes that decompose organic matter to sustain nutrient uptake directly by alfalfa. Future research should investigate the responding microbial species, their location, and especially functional traits under drought and rewetting stresses in IWG-alfalfa intercropping comparing with alfalfa and IWG sole crops.

After rewetting, the microbial biomass recovered in IWG-alfalfa intercropping, and the microbial community composition shifted just as in other cropping systems. This is probably because of the burst of available nutrients after rewetting favouring fast-growing microbes and opportunistic species, with effective osmoregulatory mechanisms, which quickly utilise the newly available resources (Hicks et al. 2022) and may outcompete the drought-resistant microbes. We do not yet know if the ecosystem function will recover after rewetting although microbial biomass recovered. The ecosystem function could be different due to the shift in microbial community composition after rewetting, as was shown by Roy et al. (2023) who reported that functional diversity is related to microbial community composition, but not to species richness. The microbial community structure has also been shown to be correlated with the resistance of respiration and fungal growth to drying, as well as bacterial resilience after rewetting (Tang et al. 2023). However, it has been suggested that the microbial function may not change even though the community composition changed due to functional redundancy (Walker 1992; Pillar et al. 2013). The response of microbial functional traits to drought and rewetting need to be studied further.

Soil microbes in perennial IWG sole cropping and IWG-alfalfa intercropping were more drought resistant and resilient than in the annual cropping systems because they had relatively more fungi-abundant microbial communities (Paper III). It has been shown that generally fungi and fungi-abundant soil food webs are more resistant to drought than bacteria and bacteria-abundant soil food webs (de Vries et al. 2018; Tang et al. 2023). Fungi may help bacteria cope with drought by forming mycelia and transferring water and nutrients (Worrich et al. 2017). High crop perenniality is associated with long-term root persistence and C and N input. Roots supply a large portion of the C used by soil microbes, and the soil microbial biomass is also part of the total amount of C in a system (Werth & Kuzyakov 2008). In a more C-rich system such as grasslands, fungi, bacteria, and their predators, fungal- and bacterial-feeding nematodes, would have a higher drought resilience than the food web in a soil where annual wheat is grown (de Vries & Bardgett 2012). In the current studies, perennial crops and soil microbes had a shared drought history. The perennial crops were established in 2016 and endured the extreme drought in 2018, while the annual crops were established in 2020 and 2021 and did not endure the dry summer in 2018 (Paper II). The historical drought can significantly influence the soil microbial responses to current drought (i.e. legacy effects) (de Vries et al. 2012; Preece et al. 2019). Possibly, the soil microbes and perennial crops could have lived together long enough to co-adapt to drought and, thus, they show higher resistance and resilience in the current rain exclusion experiment. de Vries et al. (2023) suggests that a shared drought history between plants and soil microbes is important for plant-soil feedback response to subsequent drought and that co-adaption may determine the outcome of plant-soil feedback.

4.4 The contribution of AM fungi for drought resistance and resilience of perennial sole and intercropping (Paper II and IV)

The negative correlation between grain yield loss under drought and resistance of AM fungi in Paper IV indicates the contribution of soil microbes, especially of AM fungi, to crop drought resistance. This explains the sustained yield and N content in IWG-alfalfa intercrop under drought in 2018 in Paper II. It has been shown that AM fungi form symbiotic relationships with 80-90% of vascular plant roots (Rajtor & Piotrowska-Seget 2016) and improve plant nutrient acquisition, tolerance and resilience to drought stress (Wahab et al. 2023). The interface between AM and roots allow exchanges in nutrients, signalling molecules, and protective chemical compounds (Wahab et al. 2023). AM fungi triggers plants' morphological, physiological, and molecular responses to drought stress (Wahab et al. 2023). Furthermore, AM fungi have been shown to improve plant water and nutrient uptake and use, maintain membrane integrity, protect the photosynthetic apparatus from oxidative stress, improve accumulation of osmolytes, phenols and hormones and improve the plant tolerance against drought stress (Tekaya et al. 2022). AM fungi overcome nutritional challenges to plant development by enhancing N and phosphorus uptake, and absorption of various essential vitamins (Wahab et al. 2023). In the current studies, AM fungi may have been attracted by N resources from alfalfa roots, formed symbiosis with both alfalfa and IWG in intercropping, enhanced the N uptake and water use of IWG under drought and thus sustained (Paper II) and improved (Paper IV) IWG grain yield and N content. In Paper IV, due to limited plant sample sizes and a small data set, we did not observe any correlation between AM fungal biomass and Δ^{13}C which can indicate a high water use efficiency (Bchir et al. 2016). The correlation between AM fungi biomass and Δ^{13}C needs therefore to be studied further with a larger dataset.

The highest AM fungi biomass and resilience values were observed in IWG-alfalfa intercropping under drought, indicating the facilitative interactions among IWG, alfalfa intercrop and AM fungi under drought and rewetting stress. Alfalfa and other legumes may need the symbiosis with AM fungi to acquire water and nutrients from the soil that are necessary to generate the energy required for biological N fixation, especially under drought conditions (Musyoka et al. 2020). In return, the legume provides photosynthetically produced carbohydrates to the AM fungi (Habibzadeh et al. 2013). The perennial IWG intercrop may also invest C in the common mycorrhizal network in order to exploit N rich resources brought by the alfalfa root-associated microbes. It would be interesting to quantify how much C alfalfa and IWG invest in the mycorrhizal network to enhance water and N uptake under drought.

Although the magnitude of grain yield of perennial IWG was much lower than annual wheats in the current studies and other studies (Culman et al. 2013; Dick et al. 2018; Fernandez et al. 2020; Hunter et al. 2020; Law et al. 2022), the relative yield loss of IWGs under drought was less than annuals, which is consistent with a study showing that perennial seed crops have lower but more stable yields than annual crops (Vico & Brunsell 2018). Our study showed that the capacity of perennial IWG and IWG-alfalfa to maintain stable grain yield and N content under drought was associated with higher AM fungal biomass, drought resistance, and relatively stable belowground microbial community. With more drought resistant and resilient soil microbial communities and more stable yields, perennial grain crops could potentially be more adaptable and suitable for the future climates than the current conventional annual crops.

4.5 Re-designing cropping systems with perennial intercrops for more sustainable and resilient agriculture

Our studies demonstrated the benefits of integrating legume and perenniality in current cropping system. The intercropping of perennial grain IWG and legume can improve microbial biomass and drought resilience, and provide a relatively more stable community composition compared to annual crops. Although the direct impact of this increased microbial biomass on soil biogeochemical cycles and ecosystem functions are not investigated in our current studies, we found that the increased microbial biomass contributed to soil C storage in the shallow soil layers in perennial cropping. The increased AM fungal biomass under drought contributed to higher crop yield and N stability, indicating the capability of long-term perennial grain and legume intercropping to improve agricultural soil C storage, soil health and resilience to extreme climate events.

The low grain yield compared to annual wheat is the primary reason for the current lack of adoption of perennial grain crops in food production (Chapman et al. 2022). In the current stage, it is not feasible to replace the annual wheat with current perennial IWG to feed the world. Furthermore, there are additional challenges to overcome before perennial grain crops can be implemented on a larger scale. These include maintaining yield stability over years, and reducing lodging and weed pressure. Breeding efforts with the aim of increase grain yield of IWG and other perennial grain crops are currently ongoing (DeHaan et al. 2023). The recent success of perennial rice breeding (Zhang et al. 2023) has increased expectations for a perennial agriculture. We can already integrate IWG in the current crop rotation system to enhance biodiversity and ecosystem services while progress is being made in terms of perennial IWG reaching a yield comparable to that of annual wheat. As Duchene et al. (2019) suggested IWG could be planted in marginal lands, along field edges, in lowland fields and hillsides as buffer strips to improve functional biodiversity. Meanwhile, improved knowledge of perennial crop management will support achieving a sustainable and resilient perennial agriculture. While the grain yield is important, it is not the sole consideration. Conventional monoculture of annual grain crops prioritises high grain yields at the cost of negative environmental impacts. It is clear that crop production based on conventionally managed annual sole crops is unsustainable when we consider the impact on the environment and the climate crisis. Crop diversification through increasing the level of agrobiodiversity and perenniality in cropping systems has demonstrated potential to improve ecological benefits and resilience. The ecosystem services (Figure 1) provided by perennial crop cultivation and other crop diversification practices should therefore be valued and incentivised in agricultural systems. Cropping systems need to be redesigned and transferred towards ecological intensification and to more diverse and resilient systems. There are many options to diversify cropping systems and intercropping of perennial grain and legumes is a promising tool to increase both spatial and temporal diversity, reduce the dependency on synthetic fertilizer inputs, extend the duration of soil covered by vegetation for multiple years, and thus enhance the soil biological health and resilience to climate change.

5. Conclusions

The evidence is clear: intercropping perennial grain crops and legumes delivers benefits in terms of yield and N content, as demonstrated in field and pot experiments. Management practices such as selecting appropriate companion legume species, adjusting the relative frequency of species in mixed intercrops, and regulating the level of inorganic N fertiliser can significantly influence the advantages of intercropping. The advantage of IWG and legume intercropping was most pronounced under water or N deficit conditions which are attributed to the facilitative interactions among IWG, legumes and soil microbes under abiotic stress. Specifically, IWG and legume intercrops use N more efficiently in low N input systems due to the complementarily use of N from different sources and apparent N transfer. Intercropping with alfalfa sustains grain yield and N content in IWG under drought condition due to the high drought resistance in AM fungal biomass and the more stable soil microbial community composition in intercropping. The negative correlation between drought resistance of AM fungal biomass and grain yield loss confirmed the contribution of AM fungi to crop yield stability under drought conditions. Intercropping of IWG and alfalfa also showed a high rate of recovery in microbial biomass after rewetting, probably due to the capacity of alfalfa to utilise deep soil moisture and increase root exudation of carbohydrates and N compared to IWG sole crop. Perennial grain and legume intercropping sustained yield and N content under drought conditions due to the contribution from AM fungi and microbial community structure, implying the potential of perennial intercropping to adapt to drought events caused by climate change.

Both IWG sole crop and IWG-alfalfa intercropping had higher amounts of soil microbial biomass, bacterial activity, more fungi and AM fungi abundant microbial community composition, and higher soil total C mass in the upper soil layers compared to annual crops under control treatment, due to the long-term soil cover, root exudates input, in absence of tillage practices and low N fertiliser input in perennial cropping systems. The higher microbial biomass C is linked to higher soil C concentration demonstrating the potential of perennial crops and their interactions with soil microbes to store C in soil and further mitigate climate change.

The climatic factors including precipitation, soil water content and soil temperature were the main drivers of the patterns of soil microbial abundance and temporal variations in the microbial community. At the management level, practices such as increasing the level of crop perenniality and diversity through intercropping play an important role in shaping the soil microbial community and increasing their drought resistance and resilience, which in turn improves the crop yield stability and quality.

This thesis demonstrates that IWG and alfalfa intercropping can enhance biological soil health, increase soil C storage and drought resistance and resilience in microbial biomass and community, suggesting that using perennial grain and legume intercropping in cropping system redesign is an effective strategy for climate change adaptation and mitigation to achieve a more sustainable and climate-resilient agriculture.

6. Future Perspectives

The studies in this thesis have identified the intercropping advantage in IWG and legume intercropping but the mechanisms of root-root interactions is unknown. By studying IWG and legume's roots distribution and allocation in different soil layers, we would gain valuable insights into the mechanisms behind interspecific interactions. In addition to root-root interaction, it also remains unclear how microbes interact with roots in the rhizosphere especially in perennial cropping systems under drought conditions. Furthermore, IWG and legume intercrops may facilitate each other by exchanging water and nutrients via common mycorrhizal networks. It is essential to understand how this facilitation is influenced by drought and how much C alfalfa and IWG invest respectively in the common mycorrhizal network to enhance resource uptake and exchange under drought conditions.

We observed an increase in soil total C mass in perennial cropping but the fractions and fate of this increased C are currently unknown. Studying the fractions of the increased soil total C in perennial cropping, including the contribution of perennial crop root exudates and mineralisation rates, is essential in understand the dynamics between the nutrient supply by decomposition and the C sequestration capacity provided by perennial crops. The increased C mass is associated with increased soil microbial biomass. The bacteria and fungi are a part of the soil food web, which are grazed by bacterivorous and fungivorous organisms, especially in undisturbed soils in perennial cropping. It is unclear how the predation activity influences the bacterial and fungal population and community composition. Furthermore, the interaction between microbial species under abiotic stress is a crucial area of interest. There is growing evidence that soil bacteria and fungi help each other to adapt to abiotic stress.

IWG and alfalfa intercropping harbours drought resilient microbes. By identifying these microbes at the species level, along with their location and functional traits, and by analysing substrate inputs from root exudation, we could reveal the secrets of microbial and legume alliances under drought. This will undoubtedly be helpful for isolating and culturing these drought-resistant microbes for further application.

Soil microbes have unique abilities to cope with drought and rewetting stress that go beyond the snapshots of observations using PLFA methods in this thesis. We aware the shortages of using microbial indicators to assess soil health. The correlations between soil microbial biomass, diversity and soil function are not always clear. For example, more soil microbial biomass is not necessarily optimal or desirable from agronomic perspective (Fierer et al. 2021). Furthermore, more biomass does not necessarily equate with more microbial diversity or activity (Fierer et al. 2021). The mere presence of soil pathogens does not necessarily correlate with an elevated likelihood of plant disease (Lievens et al. 2006). The species diversity of soil microbes is important but high microbial diversity does not necessarily lead to high soil function. It is widely accepted that microbial communities are characterised by a functional redundancy. Therefore it is more meaningful to view microbial communities in soil as a distribution of active sites (functional traits) rather than a distribution of species (Baveye et al. 2018). Furthermore, it is crucial to understand the location of soil microorganisms in the soil, as the physico-chemical environment and the microenvironment of microorganisms (such as “hot spots” rhizospheres and earthworm burrows, and “cold spots” usually bulk soil) influence the microbial community’s dynamics (Baveye et al. 2018). Quantifying the biomass of soil microbes and diversity is not enough. Soil needs to be viewed and studied as a system. The analysis of soil microbial communities must involve determinations of bacterial and fungal growth, distribution, function, and, if possible, the nature of interactions among species (Hill et al. 2000). To fully understand the community of soil microbes and their response abiotic stress, we must combine biological, physical and chemical indicators, and use a range of complementary tools to observe and record over time. This requires collaborations between soil biologists, soil chemists and soil physicists, as well as the integration of three subjects, soil biology, soil chemistry and soil physics as one. Analysing the biological, physical, and chemical indicators from the same soil sample is a good place to start.

References

Adl, S.M., Simpson, A.G.B., Lane, C.E., Lukeš, J., Bass, D., Bowser, S.S., Brown, M.W., Burki, F., Dunthorn, M., Hampl, V., Heiss, A., Hoppenrath, M., Lara, E., le Gall, L., Lynn, D.H., McManus, H., Mitchell, E.A.D., Mozley-Stanridge, S.E., Parfrey, L.W., Pawlowski, J., Rueckert, S., Shadwick, L., Schoch, C.L., Smirnov, A. & Spiegel, F.W. (2012). The Revised Classification of Eukaryotes. *Journal of Eukaryotic Microbiology,* 59(5), 429-514. https://doi.org/https://doi.org/10.1111/j.1550-7408.2012.00644.x

Allen, D.E., Singh, B.P. & Dalal, R.C. (2011). Soil Health Indicators Under Climate Change: A Review of Current Knowledge. In: Singh, B.P., Cowie, A.L. & Chan, K.Y. (eds) *Soil Health and Climate Change*. Springer Berlin Heidelberg. 25-45. https://doi.org/10.1007/978-3-642-20256-8_2

Allen, P., Van Dusen, D., Lundy, J. & Gliessman, S. (1991). Integrating social, environmental, and economic issues in sustainable agriculture. *American Journal of Alternative Agriculture,* 6(1), 34-39. https://doi.org/10.1017/S0889189300003787

Alletto, L., Vandewalle, A. & Debaeke, P. (2022). Crop diversification improves cropping system sustainability: An 8-year on-farm experiment in South-Western France. *Agricultural Systems,* 200, 103433. https://doi.org/https://doi.org/10.1016/j.agsy.2022.103433

Amarasinghe, H., Ratnayake, N., Ranaweera, S., Suveendran, T. & Abeyakoon, P. (2023). Qualitative analysis to identify determinants of use among different occupational settings and channels of communication to address smokeless tobacco use in Sri Lanka. *PLOS Global Public Health,* 3(1), e0001349. https://doi.org/10.1371/journal.pgph.0001349

Andrews, J.H. & Harris, R.F. (1986). r- and K-Selection and Microbial Ecology. In: Marshall, K.C. (ed.) *Advances in Microbial Ecology*. Springer US. 99-147. https://doi.org/10.1007/978-1-4757-0611-6_3

Andryukov, B.G., Karpenko, A.A. & Lyapun, I.N. (2021). Learning from Nature: Bacterial Spores as a Target for Current Technologies in Medicine (Review). *Sovrem Tekhnologii Med,* 12(3), 105-122. https://doi.org/10.17691/stm2020.12.3.13

Audu, V., Rasche, F., Dimitrova Mårtensson, L.M. & Emmerling, C. (2022). Perennial cereal grain cultivation: Implication on soil organic matter and related soil microbial parameters. *Applied Soil Ecology,* 174, 104414-104414. https://doi.org/10.1016/j.apsoil.2022.104414

Bååth, E. (1994). Measurement of protein synthesis by soil bacterial assemblages with the leucine incorporation technique. *Biology and Fertility of Soils,* 17(2), 147-153. https://doi.org/10.1007/BF00337747

Bååth, E., Pettersson, M. & Söderberg, K.H. (2001). Adaptation of a rapid and economical microcentrifugation method to measure thymidine and leucine incorporation by soil bacteria. *Soil Biology and Biochemistry,* 33(11), 1571-1574. https://doi.org/10.1016/S0038-0717(01)00073-6

Bach, E.M., Ramirez, K.S., Fraser, T.D. & Wall, D.H. (2020). Soil biodiversity integrates solutions for a sustainable future. *Sustainability,* 12(7), 2662.

Bailey, V.L., Smith, J.L. & Bolton, H. (2002). Fungal-to-bacterial ratios in soils investigated for enhanced C sequestration. *Soil Biology and Biochemistry,* 34(7), 997-1007. https://doi.org/10.1016/S0038-0717(02)00033-0

Bardgett, R.D. & van der Putten, W.H. (2014). Belowground biodiversity and ecosystem functioning. *Nature,* 515(7528), 505-511. https://doi.org/10.1038/nature13855

Barnard, R.L., Osborne, C.A. & Firestone, M.K. (2013). Responses of soil bacterial and fungal communities to extreme desiccation and rewetting. *The ISME Journal,* 7(11), 2229-2241. https://doi.org/10.1038/ismej.2013.104

Barreiro, A. & Albertsson, J. (2022). SITES Agroecological Field Experiment (SAFE).

Barreiro, A., Fontúrbel, M.T., Lombao, A., Martín, A., Vega, J.A., Fernández, C., Carballas, T. & Díaz-Raviña, M. (2015). Using phospholipid fatty acid and community level physiological profiling techniques to characterize soil microbial communities following an experimental fire and different stabilization treatments. *CATENA,* 135, 419-429. https://doi.org/10.1016/j.catena.2014.07.011

Barrios, E. (2007). Soil biota, ecosystem services and land productivity. *Ecological Economics,* 64(2), 269-285. https://doi.org/10.1016/j.ecolecon.2007.03.004

Baveye, P.C., Otten, W., Kravchenko, A., Balseiro-Romero, M., Beckers, É., Chalhoub, M., Darnault, C., Eickhorst, T., Garnier, P., Hapca, S., Kiranyaz, S., Monga, O., Mueller, C.W., Nunan, N., Pot, V., Schlüter, S., Schmidt, H. & Vogel, H.-J. (2018). Emergent Properties of Microbial Activity in Heterogeneous Soil Microenvironments: Different Research Approaches Are Slowly Converging, Yet Major Challenges Remain. *Frontiers in Microbiology,* 9. https://www.frontiersin.org/journals/microbiology/articles/10.3389/fmicb.2018.01929

Bchir, A., Escalona, J.M., Gallé, A., Hernández-Montes, E., Tortosa, I., Braham, M. & Medrano, H. (2016). Carbon isotope discrimination (δ13C) as an indicator of vine water status and water use efficiency (WUE): Looking for the most representative sample and sampling time. *Agricultural Water Management,* 167, 11-20. https://doi.org/10.1016/j.agwat.2015.12.018

Bedoussac, L., Journet, E.-P., Hauggaard-Nielsen, H., Naudin, C., Corre-Hellou, G., Jensen, E.S., Prieur, L. & Justes, E. (2015). Ecological principles underlying the increase of productivity achieved by cereal-grain legume intercrops in organic farming. A review. *Agronomy for Sustainable Development,* 35(3), 911-935. https://doi.org/10.1007/s13593-014-0277-7

Begum, N., Qin, C., Ahanger, M.A., Raza, S., Khan, M.I., Ashraf, M., Ahmed, N. & Zhang, L. (2019). Role of Arbuscular Mycorrhizal Fungi in Plant Growth Regulation: Implications in Abiotic Stress Tolerance. *Frontiers in Plant Science,* 10. https://www.frontiersin.org/journals/plant-science/articles/10.3389/fpls.2019.01068

Behrooz, A., Vahdati, K., Rejali, F., Lotfi, M., Sarikhani, S. & Leslie, C. (2019). Arbuscular Mycorrhiza and Plant Growth-promoting Bacteria Alleviate Drought Stress in Walnut. *HortScience horts,* 54(6), 1087-1092. https://doi.org/10.21273/HORTSCI13961-19

Bhowmik, A., Kukal, S.S., Saha, D., Sharma, H., Kalia, A. & Sharma, S. (2019). Potential indicators of soil health degradation in different land use-based ecosystems in the Shiwaliks of Northwestern India. *Sustainability,* 11(14), 3908.

Birch, H.F. (1958). The effect of soil drying on humus decomposition and nitrogen availability. *Plant and Soil,* 10(1), 9-31. https://doi.org/10.1007/BF01343734

Bligh, E.G. & Dyer, W.J. (1959). A RAPID METHOD OF TOTAL LIPID EXTRACTION AND PURIFICATION. *Canadian Journal of Biochemistry and Physiology,* 37(8), 911-917. https://doi.org/10.1139/o59-099

Bohlool, B.B., Ladha, J.K., Garrity, D.P. & George, T. (1992). Biological nitrogen fixation for sustainable agriculture: A perspective. *Plant and Soil,* 141(1), 1-11. https://doi.org/10.1007/BF00011307

Bossio, D.A., Cook-Patton, S.C., Ellis, P.W., Fargione, J., Sanderman, J., Smith, P., Wood, S., Zomer, R.J., von Unger, M., Emmer, I.M. & Griscom, B.W. (2020). The role of soil carbon in natural climate solutions. *Nature Sustainability,* 3(5), 391-398. https://doi.org/10.1038/s41893-020-0491-z

Bouma, J., Pinto-Correia, T. & Veerman, C. (2021). Assessing the role of soils when developing sustainable agricultural production systems focused on achieving the UN-SDGs and the EU Green Deal. *Soil Systems,* 5(3), 56.

Bünemann, E.K., Bongiorno, G., Bai, Z., Creamer, R.E., De Deyn, G., de Goede, R., Fleskens, L., Geissen, V., Kuyper, T.W., Mäder, P., Pulleman, M., Sukkel, W., van Groenigen, J.W. & Brussaard, L. (2018). Soil quality – A critical review. *Soil Biology and Biochemistry,* 120, 105-125. https://doi.org/https://doi.org/10.1016/j.soilbio.2018.01.030

Campbell, B.M., Beare, D.J., Bennett, E.M., Hall-Spencer, J.M., Ingram, J.S.I., Jaramillo, F., Ortiz, R., Ramankutty, N., Sayer, J.A. & Shindell, D. (2017). Agriculture production as a major driver of the Earth system exceeding planetary boundaries. *Ecology and Society,* 22(4). https://www.jstor.org/stable/26798991 [2024/10/17/]

Carlsson, G. & Huss-Danell, K. (2003). Nitrogen fixation in perennial forage legumes in the field. *Plant and Soil,* 253(2), 353-372. https://doi.org/10.1023/A:1024847017371

Carranca, C., de Varennes, A. & Rolston, D. (1999). Biological nitrogen fixation by fababean, pea and chickpea, under field conditions, estimated by the

15N isotope dilution technique. *European Journal of Agronomy,* 10(1), 49-56. https://doi.org/https://doi.org/10.1016/S1161-0301(98)00049-5

Chamkhi, I., Cheto, S., Geistlinger, J., Zeroual, Y., Kouisni, L., Bargaz, A. & Ghoulam, C. (2022). Legume-based intercropping systems promote beneficial rhizobacterial community and crop yield under stressing conditions. *Industrial Crops and Products,* 183, 114958. https://doi.org/https://doi.org/10.1016/j.indcrop.2022.114958

Chapman, E.A., Thomsen, H.C., Tulloch, S., Correia, P.M.P., Luo, G., Najafi, J., DeHaan, L.R., Crews, T.E., Olsson, L., Lundquist, P.-O., Westerbergh, A., Pedas, P.R., Knudsen, S. & Palmgren, M. (2022). Perennials as Future Grain Crops: Opportunities and Challenges. *Frontiers in Plant Science,* 13. https://doi.org/10.3389/fpls.2022.898769

Clément, C., Sleiderink, J., Svane, S.F., Smith, A.G., Diamantopoulos, E., Desbrøll, D.B. & Thorup-Kristensen, K. (2022). Comparing the deep root growth and water uptake of intermediate wheatgrass (Kernza®) to alfalfa. *Plant and Soil,* 472(1-2), 369-390. https://doi.org/10.1007/s11104-021-05248-6

Costa, O.Y.A., Raaijmakers, J.M. & Kuramae, E.E. (2018). Microbial Extracellular Polymeric Substances: Ecological Function and Impact on Soil Aggregation. *Frontiers in Microbiology,* 9. https://www.frontiersin.org/journals/microbiology/articles/10.3389/fmicb.2018.01636

Costanza, R., d'Arge, R., de Groot, R., Farber, S., Grasso, M., Hannon, B., Limburg, K., Naeem, S., O'Neill, R.V., Paruelo, J., Raskin, R.G., Sutton, P. & van den Belt, M. (1997). The value of the world's ecosystem services and natural capital. *Nature,* 387(6630), 253-260. https://doi.org/10.1038/387253a0

Crain, J., Wagoner, P., Larson, S. & DeHaan, L. (2024). Origin of current intermediate wheatgrass germplasm being developed for Kernza grain production. *Genetic Resources and Crop Evolution*. https://doi.org/10.1007/s10722-024-01952-1

Crews, T.E., Kemp, L., Bowden, J.H. & Murrell, E.G. (2022). How the Nitrogen Economy of a Perennial Cereal-Legume Intercrop Affects Productivity: Can Synchrony Be Achieved? *Frontiers in Sustainable Food Systems,* 6. https://www.frontiersin.org/journals/sustainable-food-systems/articles/10.3389/fsufs.2022.755548

Crippa, M., Solazzo, E., Guizzardi, D., Monforti-Ferrario, F., Tubiello, F.N. & Leip, A. (2021). Food systems are responsible for a third of global anthropogenic GHG emissions. *Nature Food,* 2(3), 198-209. https://doi.org/10.1038/s43016-021-00225-9

Culman, S.W., Snapp, S.S., Ollenburger, M., Basso, B. & DeHaan, L.R. (2013). Soil and Water Quality Rapidly Responds to the Perennial Grain Kernza Wheatgrass. *Agronomy Journal,* 105(3), 735-744. https://doi.org/10.2134/agronj2012.0273

Czaban, W., Rasmussen, J., Laursen, B.B., Vidkjær, N.H., Sapkota, R., Nicolaisen, M. & Fomsgaard, I.S. (2018). Multiple effects of secondary metabolites on amino acid cycling in white clover rhizosphere. *Soil Biology and Biochemistry,* 123, 54-63. https://doi.org/https://doi.org/10.1016/j.soilbio.2018.04.012

Dadrasi, A., Chaichi, M., Nehbandani, A., Soltani, E., Nemati, A., Salmani, F., Heydari, M. & Yousefi, A.R. (2023). Global insight into understanding wheat yield and production through Agro-Ecological Zoning. *Scientific Reports,* 13(1), 15898-15898. https://doi.org/10.1038/s41598-023-43191-x

de Oliveira, G., Brunsell, N.A., Crews, T.E., DeHaan, L.R. & Vico, G. (2020). Carbon and water relations in perennial Kernza (Thinopyrum intermedium): An overview. *Plant Science,* 295, 110279-110279. https://doi.org/10.1016/j.plantsci.2019.110279

de Vries, F., Lau, J., Hawkes, C. & Semchenko, M. (2023). Plant–soil feedback under drought: does history shape the future? *Trends in Ecology & Evolution,* 38(8), 708-718. https://doi.org/10.1016/j.tree.2023.03.001

de Vries, F.T. & Bardgett, R.D. (2012). Plant–microbial linkages and ecosystem nitrogen retention: lessons for sustainable agriculture. *Frontiers in Ecology and the Environment,* 10(8), 425-432. https://doi.org/10.1890/110162

de Vries, F.T., Griffiths, R.I., Bailey, M., Craig, H., Girlanda, M., Gweon, H.S., Hallin, S., Kaisermann, A., Keith, A.M., Kretzschmar, M., Lemanceau, P., Lumini, E., Mason, K.E., Oliver, A., Ostle, N., Prosser, J.I., Thion, C., Thomson, B. & Bardgett, R.D. (2018). Soil bacterial networks are less stable under drought than fungal networks. *Nature Communications,* 9(1), 3033-3033. https://doi.org/10.1038/s41467-018-05516-7

de Vries, F.T., Liiri, M.E., Bjørnlund, L., Setälä, H.M., Christensen, S. & Bardgett, R.D. (2012). Legacy effects of drought on plant growth and the soil food web. *Oecologia,* 170(3), 821-33. https://doi.org/10.1007/s00442-012-2331-y

de Vries, F.T., van Groenigen, J.W., Hoffland, E. & Bloem, J. (2011). Nitrogen losses from two grassland soils with different fungal biomass. *Soil Biology and Biochemistry,* 43(5), 997-1005. https://doi.org/https://doi.org/10.1016/j.soilbio.2011.01.016

DeHaan, L.R., Anderson, J.A., Bajgain, P., Basche, A., Cattani, D.J., Crain, J., Crews, T.E., David, C., Duchene, O., Gutknecht, J., Hayes, R.C., Hu, F., Jungers, J.M., Knudsen, S., Kong, W., Larson, S., Lundquist, P.-O., Luo, G., Miller, A.J., Nabukalu, P., Newell, M.T., Olsson, L., Palmgren, M., Paterson, A.H., Picasso, V.D., Poland, J.A., Sacks, E.J., Wang, S. & Westerbergh, A. (2023). Discussion: Prioritize perennial grain development for sustainable food production and environmental benefits. *Science of The Total Environment,* 895, 164975. https://doi.org/https://doi.org/10.1016/j.scitotenv.2023.164975

Deng, F. & Liang, C. (2022). Revisiting the quantitative contribution of microbial necromass to soil carbon pool: Stoichiometric control by microbes and soil. *Soil Biology and Biochemistry,* 165, 108486. https://doi.org/https://doi.org/10.1016/j.soilbio.2021.108486

Dick, C., Cattani, D. & Entz, M.H. (2018). Kernza intermediate wheatgrass (Thinopyrum intermedium) grain production as influenced by legume intercropping and residue management. *Canadian Journal of Plant Science,* 98(6), 1376-1379. https://doi.org/10.1139/cjps-2018-0146

Doran, J.W. (1996). Soil health and global sustainability. *Soil Quality is in the Hands of the Land Manager,* 45.

Doran, J.W. & Zeiss, M.R. (2000). Soil health and sustainability: managing the biotic component of soil quality. *Applied Soil Ecology,* 15(1), 3-11. https://doi.org/https://doi.org/10.1016/S0929-1393(00)00067-6

Dubey, P.K., Singh, G.S. & Abhilash, P.C. (2020). Agriculture in a Changing Climate. In: Dubey, P.K., Singh, G.S. & Abhilash, P.C. (eds) *Adaptive Agricultural Practices: Building Resilience in a Changing Climate*. Springer International Publishing. 1-10. https://doi.org/10.1007/978-3-030-15519-3_1

Duchene, O., Celette, F., Barreiro, A., Dimitrova Mårtensson, L.-M., Freschet, G.T. & David, C. (2020). Introducing Perennial Grain in Grain Crops Rotation: The Role of Rooting Pattern in Soil Quality Management. *Agronomy,* 10(9), 1254-1254. https://doi.org/10.3390/agronomy10091254

Duchene, O., Celette, F., Ryan, M.R., DeHaan, L.R., Crews, T.E. & David, C. (2019). Integrating multipurpose perennial grains crops in Western European farming systems. *Agriculture, Ecosystems & Environment,* 284, 106591-106591. https://doi.org/10.1016/j.agee.2019.106591

Echeverria, A. & Gonzalez, E.M. (2021). Root system of Medicago sativa and Medicago truncatula: drought effects on carbon metabolism. *Plant and Soil,* 463(1-2), 249-263. https://doi.org/10.1007/s11104-021-04912-1

Emery, S.M., Bell-Dereske, L., Stahlheber, K.A. & Gross, K.L. (2022). Arbuscular mycorrhizal fungal community responses to drought and nitrogen fertilization in switchgrass stands. *Applied Soil Ecology,* 169, 104218. https://doi.org/https://doi.org/10.1016/j.apsoil.2021.104218

Fagnant, L., Duchêne, O., Celette, F., David, C., Bindelle, J. & Dumont, B. (2023). Learning about the growing habits and reproductive strategy of Thinopyrum intermedium through the establishment of its critical nitrogen dilution curve. *Field Crops Research,* 291, 108802. https://doi.org/https://doi.org/10.1016/j.fcr.2022.108802

Fan, W., Tang, F., Wang, J., Dong, J., Xing, J. & Shi, F. (2023). Drought-induced recruitment of specific root-associated bacteria enhances adaptation of alfalfa to drought stress. *Frontiers in Microbiology,* 14. https://doi.org/10.3389/fmicb.2023.1114400

Farquhar, G.D., Ehleringer, J.R. & Hubick, K.T. (1989). Carbon Isotope Discrimination and Photosynthesis. *Annual Review of Plant Biology,* 40(Volume 40, 1989), 503-537. https://doi.org/https://doi.org/10.1146/annurev.pp.40.060189.002443

Fernandez, C.W., Ehlke, N., Sheaffer, C.C. & Jungers, J.M. (2020). Effects of nitrogen fertilization and planting density on intermediate wheatgrass yield. *Agronomy Journal,* 112(5), 4159-4170. https://doi.org/10.1002/agj2.20351

Fierer, N. (2017). Embracing the unknown: disentangling the complexities of the soil microbiome. *Nature Reviews Microbiology,* 15(10), 579-590. https://doi.org/10.1038/nrmicro.2017.87

Fierer, N., Bradford, M.A. & Jackson, R.B. (2007). TOWARD AN ECOLOGICAL CLASSIFICATION OF SOIL BACTERIA. *Ecology,* 88(6), 1354-1364. https://doi.org/10.1890/05-1839

Fierer, N. & Schimel, J.P. (2003). A Proposed Mechanism for the Pulse in Carbon Dioxide Production Commonly Observed Following the Rapid Rewetting of a Dry Soil. *Soil Science Society of America Journal,* 67(3), 798-805. https://doi.org/https://doi.org/10.2136/sssaj2003.7980

Fierer, N., Wood, S.A. & Bueno de Mesquita, C.P. (2021). How microbes can, and cannot, be used to assess soil health. *Soil Biology and Biochemistry,* 153, 108111. https://doi.org/https://doi.org/10.1016/j.soilbio.2020.108111

Förster, A., Hohberg, K., Rasche, F. & Emmerling, C. (2024). Nematode community structure suggests perennial grain cropping cultivation as a nature-based solution for resilient agriculture. *Journal of Sustainable Agriculture and Environment,* 3(3), e12112. https://doi.org/https://doi.org/10.1002/sae2.12112

Frostegård, A. & Bååth, E. (1996). The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. *Biology and Fertility of Soils,* 22(1-2), 59-65. https://doi.org/10.1007/BF00384433

Frostegård, Å., Bååth, E. & Tunlio, A. (1993). Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty acid analysis. *Soil Biology and Biochemistry,* 25(6), 723-730. https://doi.org/10.1016/0038-0717(93)90113-P

Frostegård, Å., Tunlid, A. & Bååth, E. (2011). Use and misuse of PLFA measurements in soils. *Soil Biology and Biochemistry,* 43(8), 1621-1625. https://doi.org/https://doi.org/10.1016/j.soilbio.2010.11.021

Gliessman, S. (2016). Transforming food systems with agroecology. *Agroecology and Sustainable Food Systems,* 40(3), 187-189. https://doi.org/10.1080/21683565.2015.1130765

Glover, J.D. (2005). The necessity and possibility of perennial grain production systems. *Renewable Agriculture and Food Systems,* 20(1), 1-4. https://doi.org/10.1079/RAF200499

Glover, J.D., Reganold, J.P., Bell, L.W., Borevitz, J., Brummer, E.C., Buckler, E.S., Cox, C.M., Cox, T.S., Crews, T.E., Culman, S.W., DeHaan, L.R., Eriksson, D., Gill, B.S., Holland, J., Hu, F., Hulke, B.S., Ibrahim, A.M.H., Jackson, W., Jones, S.S., Murray, S.C., Paterson, A.H., Ploschuk, E., Sacks, E.J., Snapp, S., Tao, D., Van Tassel, D.L., Wade, L.J., Wyse, D.L. & Xu, Y. (2010). Increased Food and Ecosystem Security via Perennial Grains. *Science,* 328(5986), 1638-1639. https://doi.org/10.1126/science.1188761

Gougoulias, C., Clark, J.M. & Shaw, L.J. (2014). The role of soil microbes in the global carbon cycle: tracking the below-ground microbial processing of plant-derived carbon for manipulating carbon dynamics in agricultural systems. *Journal of the Science of Food and Agriculture,* 94(12), 2362-2371. https://doi.org/https://doi.org/10.1002/jsfa.6577

Griffiths, B.S. & Philippot, L. (2013). Insights into the resistance and resilience of the soil microbial community. *FEMS Microbiology Reviews,* 37(2), 112-129. https://doi.org/10.1111/j.1574-6976.2012.00343.x

Grusson, Y., Wesström, I. & Joel, A. (2021a). Impact of climate change on Swedish agriculture: Growing season rain deficit and irrigation need. *Agricultural Water Management,* 251, 106858. https://doi.org/https://doi.org/10.1016/j.agwat.2021.106858

Grusson, Y., Wesström, I., Svedberg, E. & Joel, A. (2021b). Influence of climate change on water partitioning in agricultural watersheds: Examples from Sweden. *Agricultural Water Management,* 249, 106766. https://doi.org/https://doi.org/10.1016/j.agwat.2021.106766

Gu, Z., Hu, C., Gan, Y., Zhou, J., Tian, G. & Gao, L. (2024). Role of Microbes in Alleviating Crop Drought Stress: A Review. *Plants,* 13(3), 384.

Habibzadeh, Y., Pirzad, A., Zardashti, M.R., Jalilian, J. & Eini, O. (2013). Effects of Arbuscular Mycorrhizal Fungi on Seed and Protein Yield under Water-Deficit Stress in Mung Bean. *Agronomy Journal,* 105(1), 79-84. https://doi.org/https://doi.org/10.2134/agronj2012.0069

Haines-Young, R. & Potschin, M. (2010). The links between biodiversity, ecosystem services and human well-being. *Ecosystem Ecology: a new synthesis,* 1, 110-139.

Hauggaard-Nielsen, H. & Jensen, E.S. (2001). Evaluating pea and barley cultivars for complementarity in intercropping at different levels of soil N availability. *Field Crops Research,* 72(3), 185-196. https://doi.org/https://doi.org/10.1016/S0378-4290(01)00176-9

Helgason, B.L., Walley, F.L. & Germida, J.J. (2009). Fungal and Bacterial Abundance in Long-Term No-Till and Intensive-Till Soils of the Northern Great Plains. *Soil Science Society of America Journal,* 73(1), 120-127. https://doi.org/https://doi.org/10.2136/sssaj2007.0392

Henry, A., Doucette, W., Norton, J. & Bugbee, B. (2007). Changes in Crested Wheatgrass Root Exudation Caused by Flood, Drought, and Nutrient Stress. *Journal of Environmental Quality,* 36(3), 904-912. https://doi.org/https://doi.org/10.2134/jeq2006.0425sc

Hicks, L.C., Lin, S. & Rousk, J. (2022). Microbial resilience to drying-rewetting is partly driven by selection for quick colonizers. *Soil Biology and Biochemistry,* 167, 108581-108581. https://doi.org/10.1016/j.soilbio.2022.108581

Hill, G.T., Mitkowski, N.A., Aldrich-Wolfe, L., Emele, L.R., Jurkonie, D.D., Ficke, A., Maldonado-Ramirez, S., Lynch, S.T. & Nelson, E.B. (2000). Methods for assessing the composition and diversity of soil microbial communities. *Applied Soil Ecology,* 15(1), 25-36. https://doi.org/https://doi.org/10.1016/S0929-1393(00)00069-X

Høgh-Jensen, H. & Schjoerring, J.K. (1994). Measurement of biological dinitrogen fixation in grassland: Comparison of the enriched 15N dilution and the natural 15N abundance methods at different nitrogen application rates and defoliation frequencies. *Plant and Soil,* 166(2), 153-163. https://doi.org/10.1007/BF00008328

Holland, E.A. & Coleman, D.C. (1987). Litter Placement Effects on Microbial and Organic Matter Dynamics in an Agroecosystem. *Ecology,* 68(2), 425-433. https://doi.org/10.2307/1939274

Homyak, P.M., Allison, S.D., Huxman, T.E., Goulden, M.L. & Treseder, K.K. (2017). Effects of Drought Manipulation on Soil Nitrogen Cycling: A Meta-Analysis. *Journal of Geophysical Research: Biogeosciences,* 122(12), 3260-3272. https://doi.org/https://doi.org/10.1002/2017JG004146

Hoogsteen, M.J.J., Lantinga, E.A., Bakker, E.J., Groot, J.C.J. & Tittonell, P.A. (2015). Estimating soil organic carbon through loss on ignition: effects of ignition conditions and structural water loss. *European Journal of Soil Science,* 66(2), 320-328. https://doi.org/10.1111/ejss.12224

Horrigan, L., Lawrence Robert, S. & Walker, P. (2002). How sustainable agriculture can address the environmental and human health harms of industrial agriculture. *Environmental Health Perspectives,* 110(5), 445-456. https://doi.org/10.1289/ehp.02110445

Huddell, A., Ernfors, M., Crews, T., Vico, G. & Menge, D.N.L. (2023). Nitrate leaching losses and the fate of 15N fertilizer in perennial intermediate wheatgrass and annual wheat — A field study. *Science of The Total Environment,* 857, 159255. https://doi.org/https://doi.org/10.1016/j.scitotenv.2022.159255

Humphries, A.W. & Auricht, G. (2001). Breeding lucerne for Australia's southern dryland cropping environments. *Australian Journal of Agricultural Research,* 52(2), 153-169.

Hunt, H.W., Coleman, D.C., Ingham, E.R., Ingham, R.E., Elliott, E.T., Moore, J.C., Rose, S.L., Reid, C.P.P. & Morley, C.R. (1987). The detrital food web in a shortgrass prairie. *Biology and Fertility of Soils,* 3(1), 57-68. https://doi.org/10.1007/BF00260580

Hunter, M.C., Sheaffer, C.C., Culman, S.W. & Jungers, J.M. (2020). Effects of defoliation and row spacing on intermediate wheatgrass I: Grain production. *Agronomy Journal,* 112(3), 1748-1763. https://doi.org/10.1002/agj2.20128

Jain, N., Kourampi, I., Umar, T.P., Almansoor, Z.R., Anand, A., Ur Rehman, M.E., Jain, S. & Reinis, A. (2023). Global population surpasses eight billion: Are we ready for the next billion? *AIMS Public Health,* 10(4), 849-866. https://doi.org/10.3934/publichealth.2023056

Jensen, E.S. (1996). Grain yield, symbiotic N2 fixation and interspecific competition for inorganic N in pea-barley intercrops. *Plant and Soil,* 182(1), 25-38. https://doi.org/10.1007/BF00010992

Jensen, E.S., Carlsson, G. & Hauggaard-Nielsen, H. (2020). Intercropping of grain legumes and cereals improves the use of soil N resources and reduces the requirement for synthetic fertilizer N: A global-scale analysis. *Agronomy for Sustainable Development,* 40(1), 5. https://doi.org/10.1007/s13593-020-0607-x

Jørgensen, F.V., Jensen, E.S. & Schjoerring, J.K. (1999). Dinitrogen fixation in white clover grown in pure stand and mixture with ryegrass estimated by the immobilized 15N isotope dilution method. *Plant and Soil,* 208(2), 293-305. https://doi.org/10.1023/A:1004533430467

Jungers, J., Runck, B., Ewing, P.M., Maaz, T., Carlson, C., Neyhart, J., Fumia, N., Bajgain, P., Subedi, S., Sharma, V., Senay, S., Hunter, M., Cureton, C., Gutknecht, J. & Kantar, M.B. (2023). Adapting perennial grain and oilseed crops for climate resiliency. *Crop Science,* 63(4), 1701-1721. https://doi.org/https://doi.org/10.1002/csc2.20972

Kaiser, C., Franklin, O., Dieckmann, U. & Richter, A. (2014). Microbial community dynamics alleviate stoichiometric constraints during litter decay. *Ecology Letters,* 17(6), 680-690. https://doi.org/https://doi.org/10.1111/ele.12269

Kang, Y., Khan, S. & Ma, X. (2009). Climate change impacts on crop yield, crop water productivity and food security – A review. *Progress in Natural Science,* 19(12), 1665-1674. https://doi.org/https://doi.org/10.1016/j.pnsc.2009.08.001

Karlen, D.L., Ditzler, C.A. & Andrews, S.S. (2003). Soil quality: why and how? *Geoderma,* 114(3), 145-156. https://doi.org/https://doi.org/10.1016/S0016-7061(03)00039-9

Karlen, D.L., Veum, K.S., Sudduth, K.A., Obrycki, J.F. & Nunes, M.R. (2019). Soil health assessment: Past accomplishments, current activities, and future opportunities. *Soil and Tillage Research,* 195, 104365. https://doi.org/https://doi.org/10.1016/j.still.2019.104365

Karlowsky, S., Augusti, A., Ingrisch, J., Akanda, M.K.U., Bahn, M. & Gleixner, G. (2018). Drought-Induced Accumulation of Root Exudates Supports Post-drought Recovery of Microbes in Mountain Grassland. *Frontiers in Plant Science,* 9. https://www.frontiersin.org/journals/plant-science/articles/10.3389/fpls.2018.01593

Kramer, C. & Gleixner, G. (2006). Variable use of plant- and soil-derived carbon by microorganisms in agricultural soils. *Soil Biology and Biochemistry,* 38(11), 3267-3278. https://doi.org/https://doi.org/10.1016/j.soilbio.2006.04.006

Kumar, U., Sheleke, R.M. & Singh, R. (2023). Editorial: Soil-plant-microbe interactions: An innovative approach towards improving soil health and plant growth. *Frontiers in Agronomy,* 5. https://www.frontiersin.org/journals/agronomy/articles/10.3389/fagro.2023.1165328

Kundel, D., Bodenhausen, N., Jørgensen, H.B., Truu, J., Birkhofer, K., Hedlund, K., Mäder, P. & Fliessbach, A. (2020). Effects of simulated drought on biological soil quality, microbial diversity and yields under long-term conventional and organic agriculture. *FEMS Microbiology Ecology,* 96(12). https://doi.org/10.1093/femsec/fiaa205

Lamersdorf, N.P., Beier, C., Blanck, K., Bredemeier, M., Cummins, T., Farrell, E.P., Kreutzer, K., Rasmussen, L., Ryan, M., Weis, W. & Xu, Y.J. (1998). Effect of drought experiments using roof installations on acidification/nitrification of soils. *Forest Ecology and Management,* 101(1), 95-109. https://doi.org/https://doi.org/10.1016/S0378-1127(97)00128-X

Landi, L., Valori, F., Ascher, J., Renella, G., Falchini, L. & Nannipieri, P. (2006). Root exudate effects on the bacterial communities, CO2 evolution, nitrogen transformations and ATP content of rhizosphere and bulk soils. *Soil Biology and Biochemistry,* 38(3), 509-516. https://doi.org/https://doi.org/10.1016/j.soilbio.2005.05.021

Law, E.P., Wayman, S., Pelzer, C.J., DiTommaso, A. & Ryan, M.R. (2022). Intercropping red clover with intermediate wheatgrass suppresses weeds without reducing grain yield. *Agronomy Journal*, 114(1), 700-716. https://doi.org/10.1002/agj2.20914

Layek, J., Das, A., Mitran, T., Nath, C., Meena, R.S., Yadav, G.S., Shivakumar, B.G., Kumar, S. & Lal, R. (2018). Cereal+Legume Intercropping: An Option for Improving Productivity and Sustaining Soil Health. In: Meena, R.S., Das, A., Yadav, G.S. & Lal, R. (eds) *Legumes for Soil Health and Sustainable Management*. Springer Singapore. 347-386. https://doi.org/10.1007/978-981-13-0253-4_11

Lehman, R.M., Taheri, W.I., Osborne, S.L., Buyer, J.S. & Douds, D.D. (2012). Fall cover cropping can increase arbuscular mycorrhizae in soils supporting intensive agricultural production. *Applied Soil Ecology,* 61, 300-304. https://doi.org/10.1016/j.apsoil.2011.11.008

Lehmann, J., Bossio, D.A., Kögel-Knabner, I. & Rillig, M.C. (2020). The concept and future prospects of soil health. *Nature Reviews Earth & Environment,* 1(10), 544-553. https://doi.org/10.1038/s43017-020-0080-8

Li, S., Barreiro, A., Jensen, E.S., Zhang, Y. & Mårtensson, L.-M.D. (2020). Early interspecific dynamics, dry matter production and nitrogen use in Kernza (Thinopyrum intermedium) – alfalfa (Medicago sativa L.) mixed intercropping. *Acta Agriculturae Scandinavica, Section B — Soil & Plant Science,* 70(2), 165-175. https://doi.org/10.1080/09064710.2019.1686164

Li, Y., Liu, H., Huang, G., Zhang, R. & Yang, H. (2016). Nitrate nitrogen accumulation and leaching pattern at a winter wheat: summer maize cropping field in the North China Plain. *Environmental Earth Sciences,* 75(2), 118. https://doi.org/10.1007/s12665-015-4867-8

Liang, C., Schimel, J.P. & Jastrow, J.D. (2017). The importance of anabolism in microbial control over soil carbon storage. *Nature Microbiology,* 2(8), 17105. https://doi.org/10.1038/nmicrobiol.2017.105

Lievens, B., Brouwer, M., Vanachter, A.C.R.C., Cammue, B.P.A. & Thomma, B.P.H.J. (2006). Real-time PCR for detection and quantification of fungal and oomycete tomato pathogens in plant and soil samples. *Plant Science,* 171(1), 155-165. https://doi.org/https://doi.org/10.1016/j.plantsci.2006.03.009

Liu, D., Keiblinger, K.M., Leitner, S., Wegner, U., Zimmermann, M., Fuchs, S., Lassek, C., Riedel, K. & Zechmeister-Boltenstern, S. (2019). Response of Microbial Communities and Their Metabolic Functions to Drying–

Rewetting Stress in a Temperate Forest Soil. *Microorganisms,* 7(5), 129-129. https://doi.org/10.3390/microorganisms7050129

Mahmood, S., Nunes, M.R., Kane, D.A. & Lin, Y. (2023). Soil health explains the yield-stabilizing effects of soil organic matter under drought. *Soil & Environmental Health,* 1(4), 100048. https://doi.org/https://doi.org/10.1016/j.seh.2023.100048

Manzoni, S. & Katul, G. (2014). Invariant soil water potential at zero microbial respiration explained by hydrological discontinuity in dry soils. *Geophysical Research Letters,* 41(20), 7151-7158. https://doi.org/10.1002/2014GL061467

Martin-Guay, M.-O., Paquette, A., Dupras, J. & Rivest, D. (2018). The new Green Revolution: Sustainable intensification of agriculture by intercropping. *Science of The Total Environment,* 615, 767-772. https://doi.org/https://doi.org/10.1016/j.scitotenv.2017.10.024

Mattoo, R. & Gowda, M. (2022). Harnessing soil bacteria and their benefits for sustainable agriculture with changing climate. *CABI Reviews*. https://doi.org/10.1079/cabireviews202217002

McKenna, T.P., Crews, T.E., Kemp, L. & Sikes, B.A. (2020a). Community structure of soil fungi in a novel perennial crop monoculture, annual agriculture, and native prairie reconstruction. *PLOS ONE,* 15(1), e0228202-e0228202. https://doi.org/10.1371/journal.pone.0228202

McKenna, T.P., Koziol, L., Bever, J.D., Crews, T.E. & Sikes, B.A. (2020b). Abiotic and biotic context dependency of perennial crop yield. *PLOS ONE,* 15(6), e0234546-e0234546. https://doi.org/10.1371/journal.pone.0234546

Mercado-Blanco, J., Abrantes, I., Barra Caracciolo, A., Bevivino, A., Ciancio, A., Grenni, P., Hrynkiewicz, K., Kredics, L. & Proença, D.N. (2018). Belowground Microbiota and the Health of Tree Crops. *Frontiers in Microbiology,* 9. https://www.frontiersin.org/journals/microbiology/articles/10.3389/fmicb.2018.01006

Mikola, J. & Setälä, H. (1998). Productivity and Trophic-Level Biomasses in a Microbial-Based Soil Food Web. *Oikos,* 82(1), 158-168. https://doi.org/10.2307/3546926

Moebius-Clune, B.N., Moebius-Clune, D.J., Gugino, B.K., Idowu, O.J., Schindelbeck, R.R., 1180 Ristow, A.J., van Es, H.M., Thies, J.E., Shayler, H.A., McBride, M.B., Kurtz, K.S.M, 1181 Wolfe, D.W. & Abawi, G.S. (2016). *Comprehensive Assessment of Soil Health - the Cornell Framework* (third ed.2), Cornell University, Geneva, New York (2016)

Musyoka, D.M., Njeru, E.M., Nyamwange, M.M.e. & Maingi, J.M. (2020). Arbuscular mycorrhizal fungi and Bradyrhizobium co-inoculation enhances nitrogen fixation and growth of green grams (Vigna radiata L.) under water stress. *Journal of Plant Nutrition,* 43(7), 1036-1047. https://doi.org/10.1080/01904167.2020.1711940

Nicoloso, R.S., Rice, C.W., Amado, T.J.C., Costa, C.N. & Akley, E.K. (2018). Carbon saturation and translocation in a no-till soil under organic amendments. *Agriculture, Ecosystems & Environment*, 264, 73-84. https://doi.org/https://doi.org/10.1016/j.agee.2018.05.016

Ning, Q., Chen, L., Zhang, C., Ma, D., Li, D., Han, X., Cai, Z., Huang, S. & Zhang, J. (2021). Saprotrophic fungal communities in arable soils are strongly associated with soil fertility and stoichiometry. *Applied Soil Ecology*, 159, 103843. https://doi.org/https://doi.org/10.1016/j.apsoil.2020.103843

Nunes, M.R., Karlen, D.L., Veum, K.S., Moorman, T.B. & Cambardella, C.A. (2020). Biological soil health indicators respond to tillage intensity: A US meta-analysis. *Geoderma*, 369, 114335. https://doi.org/https://doi.org/10.1016/j.geoderma.2020.114335

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R. , O'hara, R.B, Simpson, G.L., Solymos, P., Stevens, M.H.H., & Wagner, H. (2013). Package "vegan". Community ecology package. See chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://cran.r-project.org/web/packages/vegan/vegan.pdf

O'Leary, M.H. (1988). Carbon Isotopes in Photosynthesis: Fractionation techniques may reveal new aspects of carbon dynamics in plants. *BioScience*, 38(5), 328-336. https://doi.org/10.2307/1310735

Oldfield, E.E., Bradford, M.A. & Wood, S.A. (2019). Global meta-analysis of the relationship between soil organic matter and crop yields. *Soil*, 5(1), 15-32.

Olsson, J., Arheimer, B., Borris, M., Donnelly, C., Foster, K., Nikulin, G., Persson, M., Perttu, A.-M., Uvo, C.B. & Viklander, M. (2016). Hydrological climate change impact assessment at small and large scales: key messages from recent progress in Sweden. *Climate*, 4(3), 39.

Olsson, P.A., Bååth, E. & Jakobsen, I. (1997). Phosphorus effects on the mycelium and storage structures of an arbuscular mycorrhizal fungus as studied in the soil and roots by analysis of Fatty Acid signatures. *Applied and Environmental Microbiology*, 63(9), 3531-3538. https://doi.org/10.1128/aem.63.9.3531-3538.1997

Olsson, P.A. & Lekberg, Y. (2022). A critical review of the use of lipid signature molecules for the quantification of arbuscular mycorrhiza fungi. *Soil Biology and Biochemistry*, 166, 108574. https://doi.org/https://doi.org/10.1016/j.soilbio.2022.108574

Orwin, K.H. & Wardle, D.A. (2004). New indices for quantifying the resistance and resilience of soil biota to exogenous disturbances. *Soil Biology and Biochemistry*, 36(11), 1907-1912. https://doi.org/10.1016/j.soilbio.2004.04.036

Parker, S.S. & Schimel, J.P. (2011). Soil nitrogen availability and transformations differ between the summer and the growing season in a California grassland. *Applied Soil Ecology*, 48(2), 185-192. https://doi.org/https://doi.org/10.1016/j.apsoil.2011.03.007

Peixoto, L., Olesen, J.E., Elsgaard, L., Enggrob, K.L., Banfield, C.C., Dippold, M.A., Nicolaisen, M.H., Bak, F., Zang, H., Dresbøll, D.B., Thorup-Kristensen, K. & Rasmussen, J. (2022). Deep-rooted perennial crops differ in capacity to stabilize C inputs in deep soil layers. *Scientific Reports,* 12(1), 5952-5952. https://doi.org/10.1038/s41598-022-09737-1

Peng, Y., Xu, H., Shi, J., Wang, Z., Lv, J., Li, L. & Wang, X. (2024). Soil microbial composition, diversity, and network stability in intercropping versus monoculture responded differently to drought. *Agriculture, Ecosystems & Environment,* 365, 108915. https://doi.org/https://doi.org/10.1016/j.agee.2024.108915

Peoples, M.B., Faizah, A., Rerkasem, B. & Herridge, D.F. (1989). Methods for evaluating nitrogen fixation by nodulated legumes in the field.

Philippot, L., Griffiths Bryan, S. & Langenheder, S. (2021). Microbial Community Resilience across Ecosystems and Multiple Disturbances. *Microbiology and Molecular Biology Reviews,* 85(2), 10.1128/mmbr.00026-20. https://doi.org/10.1128/mmbr.00026-20

Pillar, V.D., Blanco, C.C., Müller, S.C., Sosinski, E.E., Joner, F. & Duarte, L.D.S. (2013). Functional redundancy and stability in plant communities. *Journal of Vegetation Science,* 24(5), 963-974. https://doi.org/https://doi.org/10.1111/jvs.12047

Pimentel, D., Cerasale, D., Stanley, R.C., Perlman, R., Newman, E.M., Brent, L.C., Mullan, A. & Chang, D.T.-I. (2012). Annual vs. perennial grain production. *Agriculture, Ecosystems & Environment*, 161, 1-9. https://doi.org/https://doi.org/10.1016/j.agee.2012.05.025

Pinto, P., Cartoni-Casamitjana, S., Cureton, C., Stevens, A.W., Stoltenberg, D.E., Zimbric, J. & Picasso, V.D. (2022). Intercropping legumes and intermediate wheatgrass increases forage yield, nutritive value, and profitability without reducing grain yields. *Frontiers in Sustainable Food Systems,* 6. https://www.frontiersin.org/journals/sustainable-food-systems/articles/10.3389/fsufs.2022.977841

Preece, C. & Peñuelas, J. (2016). Rhizodeposition under drought and consequences for soil communities and ecosystem resilience. *Plant and Soil,* 409(1-2), 1-17. https://doi.org/10.1007/s11104-016-3090-z

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

Quideau, S.A., McIntosh, A.C., Norris, C.E., Lloret, E., Swallow, M.J. & Hannam, K. (2016). Extraction and analysis of microbial phospholipid fatty acids in soils. *JoVE (Journal of Visualized Experiments)*(114), e54360.

Rajtor, M. & Piotrowska-Seget, Z. (2016). Prospects for arbuscular mycorrhizal fungi (AMF) to assist in phytoremediation of soil hydrocarbon contaminants. *Chemosphere,* 162, 105-116. https://doi.org/https://doi.org/10.1016/j.chemosphere.2016.07.071

Rakkar, M., Jungers, J.M., Sheaffer, C., Bergquist, G., Grossman, J., Li, F. & Gutknecht, J.L.M. (2023). Soil health improvements from using a novel perennial grain during the transition to organic production. *Agriculture, Ecosystems & Environment,* 341, 108164-108164. https://doi.org/10.1016/j.agee.2022.108164

Ramankutty, N., Evan, A.T., Monfreda, C. & Foley, J.A. (2008). Farming the planet: 1. Geographic distribution of global agricultural lands in the year 2000. *Global Biogeochemical Cycles,* 22(1). https://doi.org/https://doi.org/10.1029/2007GB002952

Ramsey, P.W., Rillig, M.C., Feris, K.P., Holben, W.E. & Gannon, J.E. (2006). Choice of methods for soil microbial community analysis: PLFA maximizes power compared to CLPP and PCR-based approaches. *Pedobiologia,* 50(3), 275-280. https://doi.org/https://doi.org/10.1016/j.pedobi.2006.03.003

Ranjard, L. & Richaume, A. (2001). Quantitative and qualitative microscale distribution of bacteria in soil. *Res Microbiol,* 152(8), 707-16. https://doi.org/10.1016/s0923-2508(01)01251-7

Reilly, E.C., Gutknecht, J.L., Tautges, N.E., Sheaffer, C.C. & Jungers, J.M. (2022). Nitrogen transfer and yield effects of legumes intercropped with the perennial grain crop intermediate wheatgrass. *Field Crops Research,* 286, 108627. https://doi.org/https://doi.org/10.1016/j.fcr.2022.108627

Rodriguez, C., Carlsson, G., Englund, J.-E., Flöhr, A., Pelzer, E., Jeuffroy, M.-H., Makowski, D. & Jensen, E.S. (2020). Grain legume-cereal intercropping enhances the use of soil-derived and biologically fixed nitrogen in temperate agroecosystems. A meta-analysis. *European Journal of Agronomy,* 118, 126077. https://doi.org/https://doi.org/10.1016/j.eja.2020.126077

Roy, S., Karapurkar, J., Baidya, P., Jose, M. & Bagchi, S. (2023). Community composition, and not species richness, of microbes influences decomposer functional diversity in soil. *Soil Biology and Biochemistry,* 187, 109225. https://doi.org/https://doi.org/10.1016/j.soilbio.2023.109225

Sachet, E., Mertz, O., Le Coq, J.-F., Cruz-Garcia, G.S., Francesconi, W., Bonin, M. & Quintero, M. (2021). Agroecological Transitions: A Systematic Review of Research Approaches and Prospects for Participatory Action Methods. *Frontiers in Sustainable Food Systems,* 5. https://www.frontiersin.org/journals/sustainable-food-systems/articles/10.3389/fsufs.2021.709401

Sackett, T.E., Classen, A.T. & Sanders, N.J. (2010). Linking soil food web structure to above- and belowground ecosystem processes: a meta-analysis. *Oikos,* 119(12), 1984-1992. https://doi.org/https://doi.org/10.1111/j.1600-0706.2010.18728.x

Saeed, Q., Xiukang, W., Haider, F.U., Kučerik, J., Mumtaz, M.Z., Holatko, J., Naseem, M., Kintl, A., Ejaz, M., Naveed, M., Brtnicky, M. & Mustafa, A. (2021). Rhizosphere Bacteria in Plant Growth Promotion, Biocontrol, and Bioremediation of Contaminated Sites: A Comprehensive Review of Effects and Mechanisms. *Int J Mol Sci,* 22(19). https://doi.org/10.3390/ijms221910529

Sahu, N., Vasu, D., Sahu, A., Lal, N. & Singh, S.K. (2017). Strength of Microbes in Nutrient Cycling: A Key to Soil Health. In: Meena, V.S., Mishra, P.K., Bisht, J.K. & Pattanayak, A. (eds) *Agriculturally Important Microbes for*

Sustainable Agriculture : Volume I: Plant-soil-microbe nexus. Springer Singapore. 69-86. https://doi.org/10.1007/978-981-10-5589-8_4

Sanaullah, M., Blagodatskaya, E., Chabbi, A., Rumpel, C. & Kuzyakov, Y. (2011). Drought effects on microbial biomass and enzyme activities in the rhizosphere of grasses depend on plant community composition. *Applied Soil Ecology,* 48(1), 38-44. https://doi.org/10.1016/j.apsoil.2011.02.004

Schaeffer, S.M., Homyak, P.M., Boot, C.M., Roux-Michollet, D. & Schimel, J.P. (2017). Soil carbon and nitrogen dynamics throughout the summer drought in a California annual grassland. *Soil Biology and Biochemistry,* 115, 54-62. https://doi.org/10.1016/j.soilbio.2017.08.009

Schimel, J., Balser, T.C. & Wallenstein, M. (2007). MICROBIAL STRESS-RESPONSE PHYSIOLOGY AND ITS IMPLICATIONS FOR ECOSYSTEM FUNCTION. *Ecology,* 88(6), 1386-1394. https://doi.org/https://doi.org/10.1890/06-0219

Schofield, R.K. & Taylor, A.W. (1955). The Measurement of Soil pH. *Soil Science Society of America Journal,* 19(2), 164-167. https://doi.org/10.2136/sssaj1955.03615995001900020013x

Shang, Y., Olesen, J.E., Lærke, P.E., Manevski, K. & Chen, J. (2024). Perennial cropping systems increased topsoil carbon and nitrogen stocks over annual systems—a nine-year field study. *Agriculture, Ecosystems & Environment,* 365, 108925. https://doi.org/https://doi.org/10.1016/j.agee.2024.108925

Shearer, G. & Kohl, D.H. (1986). N2-fixation in field settings: estimations based on natural 15N abundance. *Functional Plant Biology,* 13(6), 699-756.

Singh, D., Thapa, S., Singh, J.P., Mahawar, H., Saxena, A.K., Singh, S.K., Mahla, H.R., Choudhary, M., Parihar, M., Choudhary, K.B. & Chakdar, H. (2024). Prospecting the Potential of Plant Growth-Promoting Microorganisms for Mitigating Drought Stress in Crop Plants. *Current Microbiology,* 81(3), 84. https://doi.org/10.1007/s00284-023-03606-4

Singh, S., Mayes, M.A., Shekoofa, A., Kivlin, S.N., Bansal, S. & Jagadamma, S. (2021). Soil organic carbon cycling in response to simulated soil moisture variation under field conditions. *Scientific Reports,* 11(1), 10841-10841. https://doi.org/10.1038/s41598-021-90359-4

Six, J., Frey, S.D., Thiet, R.K. & Batten, K.M. (2006). Bacterial and Fungal Contributions to Carbon Sequestration in Agroecosystems. *Soil Science Society of America Journal,* 70(2), 555-569. https://doi.org/10.2136/sssaj2004.0347

Söderberg, K.H. & Bååth, E. (1998). Bacterial activity along a young barley root measured by the thymidine and leucine incorporation techniques. *Soil Biology and Biochemistry,* 30(10-11), 1259-1268. https://doi.org/10.1016/S0038-0717(98)00058-3

Soto-Gómez, D. & Pérez-Rodríguez, P. (2022). Sustainable agriculture through perennial grains: Wheat, rice, maize, and other species. A review. *Agriculture, Ecosystems & Environment,* 325, 107747-107747. https://doi.org/10.1016/j.agee.2021.107747

Sprunger, C.D., Culman, S.W., Peralta, A.L., DuPont, S.T., Lennon, J.T. & Snapp, S.S. (2019). Perennial grain crop roots and nitrogen management shape soil food webs and soil carbon dynamics. *Soil Biology and Biochemistry,* 137, 107573-107573. https://doi.org/10.1016/j.soilbio.2019.107573

Sun, B., Jia, S., Zhang, S., McLaughlin, N.B., Liang, A., Chen, X., Liu, S. & Zhang, X. (2016). No tillage combined with crop rotation improves soil microbial community composition and metabolic activity. *Environmental Science and Pollution Research,* 23(7), 6472-6482. https://doi.org/10.1007/s11356-015-5812-9

Tang, Y., Winterfeldt, S., Brangarí, A.C., Hicks, L.C. & Rousk, J. (2023). Higher resistance and resilience of bacterial growth to drought in grasslands with historically lower precipitation. *Soil Biology and Biochemistry,* 177, 108889. https://doi.org/https://doi.org/10.1016/j.soilbio.2022.108889

Tautges, N.E., Jungers, J.M., DeHaan, L.R., Wyse, D.L. & Sheaffer, C.C. (2018). Maintaining grain yields of the perennial cereal intermediate wheatgrass in monoculture v. bi-culture with alfalfa in the Upper Midwestern USA. *The Journal of Agricultural Science,* 156(6), 758-773. https://doi.org/10.1017/S0021859618000680

Tavi, N.M., Martikainen, P.J., Lokko, K., Kontro, M., Wild, B., Richter, A. & Biasi, C. (2013). Linking microbial community structure and allocation of plant-derived carbon in an organic agricultural soil using 13CO2 pulse-chase labelling combined with 13C-PLFA profiling. *Soil Biology and Biochemistry,* 58, 207-215. https://doi.org/10.1016/j.soilbio.2012.11.013

Taylor, K., Samaddar, S., Schmidt, R., Lundy, M. & Scow, K. (2023). Soil carbon storage and compositional responses of soil microbial communities under perennial grain IWG vs. annual wheat. *Soil Biology and Biochemistry,* 184, 109111-109111. https://doi.org/10.1016/j.soilbio.2023.109111

Taylor, K.M., Nelsen, T.S., Scow, K.M. & Lundy, M.E. (2024). No-till annual wheat increases plant productivity, soil microbial biomass, and soil carbon stabilization relative to intermediate wheatgrass in a Mediterranean climate. *Soil and Tillage Research,* 235, 105874-105874. https://doi.org/10.1016/j.still.2023.105874

Tekaya, M., Dabbaghi, O., Guesmi, A., Attia, F., Chehab, H., Khezami, L., Algathami, F.K., Ben Hamadi, N., Hammami, M., Prinsen, E. & Mechri, B. (2022). Arbuscular mycorrhizas modulate carbohydrate, phenolic compounds and hormonal metabolism to enhance water deficit tolerance of olive trees (Olea europaea). *Agricultural Water Management,* 274, 107947-107947. https://doi.org/10.1016/j.agwat.2022.107947

Testa, S., Nielsen, K.R., Vallentin, S. & Ciccullo, F. (2022). Sustainability-oriented innovation in the agri-food system: Current issues and the road ahead. *Technological Forecasting and Social Change,* 179, 121653. https://doi.org/https://doi.org/10.1016/j.techfore.2022.121653

Thilakarathna, M.S., Papadopoulos, Y.A., Rodd, A.V., Grimmett, M., Fillmore, S.A.E., Crouse, M. & Prithiviraj, B. (2016). Nitrogen fixation and transfer of red clover genotypes under legume–grass forage based production systems. *Nutrient Cycling in Agroecosystems,* 106(2), 233-247. https://doi.org/10.1007/s10705-016-9802-1

Tsiafouli, M.A., Thebault, E., Sgardelis, S.P., de Ruiter, P.C., van der Putten, W.H., Birkhofer, K., Hemerik, L., de Vries, F.T., Bardgett, R.D., Brady, M.V., Bjornlund, L., Jorgensen, H.B., Christensen, S., Hertefeldt, T.D., Hotes, S., Gera Hol, W.H., Frouz, J., Liiri, M., Mortimer, S.R., Setala, H., Tzanopoulos, J., Uteseny, K., Pizl, V., Stary, J., Wolters, V. & Hedlund, K. (2015). Intensive agriculture reduces soil biodiversity across Europe. *Glob Chang Biol,* 21(2), 973-85. https://doi.org/10.1111/gcb.12752

Ulrich, D.E.M., Sevanto, S., Ryan, M., Albright, M.B.N., Johansen, R.B. & Dunbar, J.M. (2019). Plant-microbe interactions before drought influence plant physiological responses to subsequent severe drought. *Scientific Reports,* 9(1), 249. https://doi.org/10.1038/s41598-018-36971-3

Unkovich, M., Herridge, D., Peoples, M., Cadisch, G., Boddey, B., Giller, K., Alves, B. & Chalk, P. (2008). *Measuring plant-associated nitrogen fixation in agricultural systems*. Australian Centre for International Agricultural Research (ACIAR).

van Dijk, M., Morley, T., Rau, M.L. & Saghai, Y. (2021). A meta-analysis of projected global food demand and population at risk of hunger for the period 2010–2050. *Nature Food,* 2(7), 494-501. https://doi.org/10.1038/s43016-021-00322-9

Van Duivenbooden, N., Pala, M., Studer, C., Bielders, C.L. & Beukes, D.J. (2000). Cropping systems and crop complementarity in dryland agriculture to increase soil water use efficiency: a review. *NJAS: Wageningen Journal of Life Sciences,* 48(3), 213-236. https://doi.org/10.1016/S1573-5214(00)80015-9

Vestberg, M., Palojärvi, A., Pitkänen, T., Kaipainen, S., Puolakka, E. & Keskitalo, M. (2012). Neutral lipid fatty acid analysis is a sensitive marker for quantitative estimation of arbuscular mycorrhizal fungi in agricultural soil with crops of different mycotrophy. *Agricultural and Food Science,* 21(1), 12-27. https://doi.org/10.23986/afsci.4996

Vico, G. & Brunsell, N.A. (2018). Tradeoffs between water requirements and yield stability in annual vs. perennial crops. *Advances in Water Resources,* 112, 189-202. https://doi.org/10.1016/j.advwatres.2017.12.014

Wahab, A., Muhammad, M., Munir, A., Abdi, G., Zaman, W., Ayaz, A., Khizar, C. & Reddy, S.P.P. (2023). Role of arbuscular mycorrhizal fungi in regulating growth, enhancing productivity, and potentially influencing ecosystems under abiotic and biotic stresses. *Plants,* 12(17), 3102.

Walker, B.H. (1992). Biodiversity and Ecological Redundancy. *Conservation Biology,* 6(1), 18-23. http://www.jstor.org/stable/2385847 [2024/10/17/]

Wallander, H., Ekblad, A., Godbold, D.L., Johnson, D., Bahr, A., Baldrian, P., Björk, R.G., Kieliszewska-Rokicka, B., Kjøller, R., Kraigher, H., Plassard, C. & Rudawska, M. (2013). Evaluation of methods to estimate production, biomass and turnover of ectomycorrhizal mycelium in forests soils – A review. *Soil Biology and Biochemistry,* 57, 1034-1047. https://doi.org/https://doi.org/10.1016/j.soilbio.2012.08.027

Wander, M.M., Cihacek, L.J., Coyne, M., Drijber, R.A., Grossman, J.M., Gutknecht, J.L.M., Horwath, W.R., Jagadamma, S., Olk, D.C., Ruark, M., Snapp, S.S., Tiemann, L.K., Weil, R. & Turco, R.F. (2019). Developments in Agricultural Soil Quality and Health: Reflections by the Research Committee on Soil Organic Matter Management. *Frontiers in Environmental Science,* 7. https://www.frontiersin.org/journals/environmental-science/articles/10.3389/fenvs.2019.00109

Wardle, D.A., Bardgett, R.D., Klironomos, J.N., Setälä, H., van der Putten, W.H. & Wall, D.H. (2004). Ecological Linkages Between Aboveground and Belowground Biota. *Science,* 304(5677), 1629-1633. https://doi.org/10.1126/science.1094875

Watzinger, A. (2015). Microbial phospholipid biomarkers and stable isotope methods help reveal soil functions. *Soil Biology and Biochemistry,* 86, 98-107. https://doi.org/https://doi.org/10.1016/j.soilbio.2015.03.019

Webb, B.A., Hildreth, S., Helm, R.F. & Scharf, B.E. (2014). Sinorhizobium meliloti Chemoreceptor McpU Mediates Chemotaxis toward Host Plant Exudates through Direct Proline Sensing. *Applied and Environmental Microbiology,* 80(11), 3404-3415. https://doi.org/10.1128/AEM.00115-14

Werth, M. & Kuzyakov, Y. (2008). Root-derived carbon in soil respiration and microbial biomass determined by 14C and 13C. *Soil Biology and Biochemistry,* 40(3), 625-637. https://doi.org/10.1016/j.soilbio.2007.09.022

Willers, C., Jansen van Rensburg, P.J. & Claassens, S. (2015). Phospholipid fatty acid profiling of microbial communities–a review of interpretations and recent applications. *Journal of Applied Microbiology,* 119(5), 1207-1218. https://doi.org/https://doi.org/10.1111/jam.12902

Willey, R.W. (1979). Intercropping - its importance and research needs. 1. Competition and yield advantages. *Field Crop Abstracts,* 32(1), 1–10.

Willey, R.W. & Rao, M.R. (1980). A Competitive Ratio for Quantifying Competition Between Intercrops. *Experimental Agriculture,* 16(2), 117-125. https://doi.org/10.1017/S0014479700010802

Wittwer, R.A., Bender, S.F., Hartman, K., Hydbom, S., Lima, R.A.A., Loaiza, V., Nemecek, T., Oehl, F., Olsson, P.A., Petchey, O., Prechsl, U.E., Schlaeppi, K., Scholten, T., Seitz, S., Six, J. & van der Heijden, M.G.A. (2021). Organic and conservation agriculture promote ecosystem multifunctionality. *Science Advances,* 7(34), eabg6995. https://doi.org/doi:10.1126/sciadv.abg6995

Worrich, A., Stryhanyuk, H., Musat, N., König, S., Banitz, T., Centler, F., Frank, K., Thullner, M., Harms, H., Richnow, H.-H., Miltner, A., Kästner, M. & Wick, L.Y. (2017). Mycelium-mediated transfer of water and nutrients stimulates bacterial activity in dry and oligotrophic environments. *Nature Communications,* 8(1), 15472. https://doi.org/10.1038/ncomms15472

Wu, H., Cui, H., Fu, C., Li, R., Qi, F., Liu, Z., Yang, G., Xiao, K. & Qiao, M. (2024). Unveiling the crucial role of soil microorganisms in carbon cycling: A review. *Science of The Total Environment*, 909, 168627. https://doi.org/https://doi.org/10.1016/j.scitotenv.2023.168627

Xu, X., Sharma, P., Shu, S., Lin, T.-S., Ciais, P., Tubiello, F.N., Smith, P., Campbell, N. & Jain, A.K. (2021). Global greenhouse gas emissions from animal-based foods are twice those of plant-based foods. *Nature Food,* 2(9), 724-732. https://doi.org/10.1038/s43016-021-00358-x

Yin, W., Chai, Q., Zhao, C., Yu, A., Fan, Z., Hu, F., Fan, H., Guo, Y. & Coulter, J.A. (2020). Water utilization in intercropping: A review. *Agricultural Water Management,* 241, 106335. https://doi.org/https://doi.org/10.1016/j.agwat.2020.106335

Yu, H., Liu, X., Ma, Q., Yin, Z., Wang, Y., Xu, Z. & Zhou, G. (2021). Climatic warming enhances soil respiration resilience in an arid ecosystem. *Science of The Total Environment,* 756, 144005. https://doi.org/https://doi.org/10.1016/j.scitotenv.2020.144005

Zhang, S., Huang, G., Zhang, Y., Lv, X., Wan, K., Liang, J., Feng, Y., Dao, J., Wu, S., Zhang, L., Yang, X., Lian, X., Huang, L., Shao, L., Zhang, J., Qin, S., Tao, D., Crews, T.E., Sacks, E.J., Lyu, J., Wade, L.J. & Hu, F. (2023). Sustained productivity and agronomic potential of perennial rice. *Nature Sustainability,* 6(1), 28-38. https://doi.org/10.1038/s41893-022-00997-3

Zhang, X., Zhang, Y., Sha, L., Wu, C., Tan, Z., Song, Q., Liu, Y. & Dong, L. (2015). Effects of continuous drought stress on soil respiration in a tropical rainforest in southwest China. *Plant and Soil,* 394(1), 343-353. https://doi.org/10.1007/s11104-015-2523-4

Popular science summary

Imagine a world where crops grow year after year without the need for replanting and where chemical fertilizers are almost unnecessary. These crops have deep roots that access water and nutrients deep in the soil, creating a thriving environmental for soil microbes, insects, and wildlife, even amidst a changing climate. Farmers benefit from stable, resilient crops that naturally enrich the soil, while consumers enjoy grain products that are good for their health as well as for the planet. This is not a fantasy but the potential of using perennial grain crops. Perennial crops, which do not need to be replanted each season, are being developed and tested worldwide. In China, for example, a perennial rice has been developed, with yield similar to regular rice but with greater environmental and economic benefits. My research focuses on "Kernza®" intermediate wheatgrass, the first perennial grain that was developed and commercialised. I studied how Kernza responds to drought compared to annual cereals in sourthern Sweden, focusing on soil microbes because they play a crucial role in supporting plant growth and adaptation to stress. Over two years, I compared soil microbial populations and communities in Kernza sole cropping, Kernza-legume intercropping, and annual cereal sole cropping under simulated drought by reducing rainfall in field. The results are promising. When Kernza was intercropped with the legume alfalfa, it supported more beneficial soil microbes, like mycorrhizal fungi, which help plants access water and nutrient even during dry spells. This partnership among Kernza, alfalfa and arbuscular mycorrhizal fungi stabilized Kernza's grain yield and improved its nitrogen nutrient uptake under drought conditions. Furthermore, Kernza helped store more carbon in the upper soil layers, a key factor in mitigating climate change. Perennial grains like Kernza provide a solution to improve soil health and increase resilience to drought, paving a way toward more sustainable agriculture and a healthier planet.

Populärvetenskaplig sammanfattning

Föreställ dig en värld där grödor växer år efter år utan att behöva återplanteras och där behovet av kemiska gödselmedel är minimalt. Dessa grödor har djupa rötter som når vatten och näringsämnen långt ner i marken, vilket skapar en gynnsam miljö för markmikrober, insekter och vilda djur, även under förändrade klimatförhållanden. Lantbrukare får stabila skördar tack vare motståndskraftiga grödor som berikar jorden, medan konsumenterna kan njuta av hälsosamma spannmålsprodukter. Detta är potentialen hos fleråriga spannmålsgrödor, som inte behöver återplanteras efter varje säsong. De har utvecklats och testas världen över, exempelvis har det tagit fram ett flerårigt ris i Kina med liknande avkastning som vanligt ris men med större miljömässiga och ekonomiska fördelar. Min forskning fokuserar på Kernza®, den första fleråriga spannmålsgrödan som har utvecklats och kommersialiserats. Jag studerade hur Kernza klarar torka jämfört med ettåriga spannmål i södra Sverige, med fokus på markmikrober, som är avgörande för växters tillväxt och stressanpassning. Under två år jämförde jag markmikrobers populationsstorlek och sammansättning i Kernza-monokultur, Kernza-lusern-samodlad, och ettåriga spannmålsgrödor under simulerad torka. Resultaten visade att jorden där Kernza odlades med lusern innehöll fler gynnsamma markmikrober, som mykorrhizasvampar, som hjälper växter att ta upp vatten och näringsämnen under torra perioder. Detta samarbete stabiliserade Kernza’s avkastning och förbättrade dess kväveupptag under torka, och bidrog till att lagra mer kol i de övre markskikten—en viktig faktor för att motverka klimatförändringarna. Fleråriga spannmål som Kernza erbjuder en lösning för att förbättra jordhälsan och öka återhämtningsförmåga från torka, vilket banar vägen för en mer hållbart jordbruk och en friskare planet.

Acknowledgements

The completion of this thesis would not have been possible without the support, guidance, and encouragement from many people. First and foremost, I would like to thank my supervisor, Linda-Maria Dimitrova Mårtensson, for her constant belief in me, insightful feedback, and invaluable advice that shaped both this work and my growth as a young scholar. She always told me that be humble and clear. I would also like to thank my co-supervisors, Ana Barreiro, for teaching me the PLFA methods and GC analyses and always being there for discussions about anything that I was unsure on. Thomas Prade, for providing valuable guidance and feedback on my manuscripts, presentations, posters and this thesis. I must thank Juan Almeida for outstanding feedback on manuscripts and this thesis, and valuable discussions that broaden my perspective on this topic. I am grateful to our emeritus professor Erik Steen Jensen for introducing me to the perennial crop topic and gave me the opportunity to come to Sweden in the very first place. Thank Prof. Yingjun Zhang and Nan Liu for their support and help to make my first visiting in Sweden possible. My heartfelt thanks go to my colleagues at SCS group; Ryan Davidson, Eamon Gallagher, Johan Sollerhed, for helping me drill very dry and compact soil to collect samples. Maria Grudén, Lina Fransson Engman, Linda Groot Nibbelink for helping with milling and weighing plant and soil samples. Bindu Sunilkumar for helping with C N analyses. Dennis Ried and Elsa Svensson for helping with pH analyses. Erik Rasmusson and Johannes Albertsson for always answering my questions in detail. I am also thankful to Ida Lager and Kamil Demski from plant breeding department for their guidance and assistance on GC analyses. Johannes Rousk for helping with radioactivity analyses in Lund University. Thanks to Helene Larsson Jönsson for taking care of my PhD administration. Many thanks to Maria Hellström for booking all the technical support for my dissertation and always so helpful and kind. Georg Carlsson for his insightful feedback on my presentations and this thesis. Maria Ernfors, Raj Chongtham and Hanna Williams for their feedback on my presentations. Dylan Wallman for his good advice on work life balance in Sweden. Thanks to Isabella Kleman, Alejandro Barrios Latorre, Satyajeet Gupta, Yuzhou Lan and Ying Liu for their good tips on PhD courses, conferences, and travel grants application. I owe the deepest gratitude to my family. To my parents and my brother (李有福, 晋成莲, 李守国), thank you for your unconditional love and endless support（谢谢你们无私的爱和支持). To all my relatives in China, thank you for your care and concern for me all the time. To my dear departed grandma, thank for your unwavering belief in me when I was a child, I strive every day to make you proud. To Ia Lindgren Säwemark and Hans Lindgren, thank you for your encouragement and support during my PhD. A special thank you to my partner, Lucas Lindgren, your love and encouragement gave me the strength to keep going through the most challenging moments. Lastly, I would like to acknowledge BiodivERsA 2019-2020 under the BiodivClim ERA-Net COFUND programme, Crafoord Foundation 20160622 and Department of Biosystems and Technology for their financial support for this PhD project. I acknowledge SITES (funded by the Swedish Research Council 2017-00635) for providing research platform. I also appreciate Alnarp park and the gym Gerdahallen for keeping me healthy during the stressful thesis writing periods. Thank you all for your support and help!

agronomy

Article

Species Interactions and Nitrogen Use during Early Intercropping of Intermediate Wheatgrass with a White Clover Service Crop

Shoujiao Li [1], Erik Steen Jensen [1], Nan Liu [2], Yingjun Zhang [2,*] and Linda-Maria Dimitrova Mårtensson [1,*]

[1] Department of Biosystems and Technology, Swedish University of Agricultural Sciences, P.O. Box 103, 230 53 Alnarp, Sweden; shoujiao.li@slu.se (S.L.); erik.steen.jensen@slu.se (E.S.J.)

[2] College of Grassland Sciences and Technology, China Agricultural University, Beijing 100193, China; liunan@cau.edu.cn

* Correspondence: zhangyj@cau.edu.cn (Y.Z.); linda.maria.martensson@slu.se (L.-M.D.M.); Tel.: +86-13611272283 (Y.Z.); +46-722385816 (L.-M.D.M.)

Abstract: Perennial grain crops intercropped with legumes are expected to use nitrogen (N) resources efficiently. A pot experiment using the ^{15}N isotope dilution method demonstrated interspecific competition and use of N from the soil and N_2 fixation in intermediate wheatgrass (*Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey, IWG) and white clover (*Trifolium repens* L., WC) intercrops at five species-relative frequencies and four levels of inorganic N fertilizer in a replacement series design. The proportion of N in WC derived from the atmosphere increased from 39.7% in a sole crop to 70.9% when intercropped with IWG, and 10.1% N in IWG transferred from WC. Intermediate wheatgrass showed high fitness with maintained high total dry matter production at low relative frequencies. Decreasing IWG-relative frequency only increased dry matter and N accumulation of WC, resulting in increased amounts of N_2 fixed. Increased levels of N fertilization increased the proportion of N acquired from the fertilizer in IWG and WC but decreased the N fixed by WC and N absorbed by IWG from the soil. Our study indicates that WC supply sufficient fixed N_2 for IWG intercrop biomass yields under appropriate levels of soil N fertility and species-relative frequencies.

Keywords: symbiotic N_2 fixation; apparent transfer of N; intercropping advantages; interspecific interactions; intermediate wheatgrass; white clover; service crop

Citation: Li, S.; Jensen, E.S.; Liu, N.; Zhang, Y.; Dimitrova Mårtensson, L.-M. Species Interactions and Nitrogen Use during Early Intercropping of Intermediate Wheatgrass with a White Clover Service Crop. *Agronomy* **2021**, *11*, 388. https://doi.org/10.3390/agronomy11020388

Academic Editors: Gabriel de Oliveira, Nathaniel A. Brunsell, Timothy E. Crews and Lee R. DeHaan

Received: 25 January 2021
Accepted: 18 February 2021
Published: 22 February 2021

1. Introduction

Agriculture is under increasing pressure to improve productivity while limiting negative environmental impacts under the circumstances of climate change and population growth. The current global agriculture is dominated by the cultivation of annual crops, which may lead to many environmental problems, due to practices such as frequent tillage, reduced soil organic matter, and overuse of fertilizers and pesticides [1]. Perennial grain crops have been proposed by scientists to reduce these problems. They have extensive root systems and several years of permanent ground cover, which could increase water and nutrient use efficiency, soil organic matter, carbon sequestration, soil faunal diversity, and decrease tillage, soil erosion, and energy consumption [2]. Kernza is the first commercial perennial grain crop in the world, domesticated from the forage grass species intermediate wheatgrass (IWG) (*Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey) [3]. The grain yield of IWG is currently much lower than that of annual wheat, but international breeding programs are working to increase yields. Despite the modest grain yields, organic and conventional farmers in France and the United States are interested in growing perennial grains for the reasons of increasing or maintaining farm profitability and improving soil health [4]. The capacity of IWG to reduce and prohibit nitrate leaching is confirmed by several studies [3,5]. Intermediate wheatgrass can be used to produce both grain and forage to provide additional economic benefits. Intercropping IWG with legumes has also been suggested to improve the feasibility of perennial grain cultivation [6]. The intercropping of cereals and legume service crops has been approved to produce greater yields, improve nutrient use efficiency, improve soil fertility through biological N_2 fixation, provide better lodging resistance, reduce pest incidence, improve forage quality, save synthetic fertilizer use, thus offering greater financial stability as compared to sole crops grown on the same amount of land [7,8].

The introduction of a legume service crop provides diverse ecosystem services to the intercropping systems [9,10]; however, it could also induce competition between the legume and cereal components. Choosing an appropriate companion legume service crop is important for establishing a stable mixture. Since perennial cereals are relatively new crops, limited research on intercropping with legumes has been performed. Hayes et al. [11] found that alternate rows of perennial wheat (*Thinopyrum* spp. × *Triticum aestivum* L.) lines and subterranean clover (*Trifolium subterraneum* L.) intercropping increased subterranean clover biomass and regeneration, and subterranean clover fixed sufficient N to maintain the N balance of a cropping system producing 1.5–2.0 t cereal grain ha^{-1} each year. Intercropping of alfalfa (*Medicago sativa*) and IWG had similar IWG yields and nutrient acquisition and lower yield declines than the IWG sole crop fertilized with N in the Upper Midwestern USA [12]. Intercropping red clover (*Trifolium pratense* L.) with IWG consistently increased the nutritive value of the summer and fall forage of the first-year IWG and red clover stand in southern Wisconsin USA [13]. Our previous study showed that alfalfa was very aggressive when intercropped with IWG [14]. A similar result was also observed by Dick et al. [6], where alfalfa became dominant in alfalfa and IWG mixed pastures, thus, the IWG biomass was negatively influenced. However, IWG performed best when intercropping with white clover (*Trifolium repens* L., WC) compared to alfalfa or sweet clover (*Melilotus officinalis*) in the dual-purpose IWG system, because the prostrate growth habit and patterns of rooting depth of WC led to weak competition for light and nutrient [6,15]. The higher grain yields of IWG when grown in association with WC than when grown in monoculture were found in a field experiment at the Rodale Institute [15,16].

Furthermore, a higher rate of N_2 fixation [17] and N transfer [18–20] was reported from WC to neighboring plants compared to red clover or alfalfa. Literature data also show that up to 545 kg N ha^{-1} $year^{-1}$ can be fixed by WC above-ground biomass in un-grazed northern temperate/boreal areas [17]. From 0 to 73% nitrogen could apparently be transferred from forage legumes to companion grasses in mixed stands, after one to four production years [21]. The ^{15}N isotope dilution method is one of the commonly used methods for the measurements of N_2 fixation. Soils often show slightly higher ^{15}N abundance than atmospheric N_2 does, due to the isotopic discrimination during biological, chemical, and physical processes [17]. This small difference can be utilized to distinguish between legume N derived from the soil and air, respectively. The higher ^{15}N abundance of soil derived N in legumes has been diluted by the low ^{15}N abundance of atmospheric N_2 as symbiotic N_2 fixation happened. A reference plant that relies only on soil nitrogen is used to estimate the ^{15}N abundance of soil N utilized by the legume. Artificially enriched ^{15}N fertilizer can be added to the soil to enlarge the difference between the ^{15}N composition of soil and the atmosphere [22]. Thus, the difference in ^{15}N abundances between the legume and the reference plant will be greater, allowing for precise estimations of N_2 fixation. A difficulty with this ^{15}N isotope dilution method is that the reference plant should have a similar pattern of N uptake as the legume and exploit the same soil N pool in order to obtain soil N of the same ^{15}N enrichment as the legume [17,22]. It is, therefore, important to make sure the legume and the reference plant utilize soil N from the same soil depth and at the same time, and the added ^{15}N is distributed evenly with soil depth and time.

In a legume and cereal intercropping system, the intercropping advantage can be influenced by both plant density and relative frequency of the intercrop components [23]. Relative frequency is the number of occurrences of a named species divided by the total occurrence of all species times one hundred [24]. Lithourgidis et al. [25] found that the relative yield total of the common vetch and oat mixtures exhibited an increasing trend as the common vetch proportion increased. Arlauskiene et al. [26] found that cereal aggressivity in the pea/barley, pea/oats, and pea/triticale intercrops depended on pea density, and in the pea/barley intercrops with an increasing number of pea plants, the competitive ratio of barley declined. Thus, the relative frequency of intercrop components could alter the outcome of competitive dynamics between component species and determine yields and production efficiency of cereal and legume intercropping systems. Up to now, how species-relative frequency might influence the interspecific competition, intercropping advantages, and yields of IWG and the service crop WC remains unknown.

Soil inorganic N concentration is also an important factor in determining intercropping advantages and interspecific interactions in a legume and cereal intercrop system [27]. Numerous studies have shown that the intercrop advantage is more evident on soils with low N availability, and it is significantly reduced by higher N input [26–29]. Intercropping advantages in cereal–legume intercrop are obtained, mainly due to the niche segregation for N resources between legumes and cereals [8,27] and potential N transfer from the legume to the cereal after some years [30].

Previous studies about N fertilization in the IWG cropping system mainly focused on the effects of N fertilizer on grain and biomass yields of IWG sole crops [31–33]. Jungers et al. [32] found that there was a quadratic response of IWG grain yield to increasing levels of N fertilizer where the optimal N rate range is 61 to 96 kg N ha^{-1}. Fernandez et al. [33] found that grain and biomass of IWG response to N fertilization were greatest in years 2 and 3. Tautges et al. [12] reported that N fertilization increased grain yield of IWG in year 2 but did not mitigate the decline in yields as stands aged. However, there is little published information on the effects of N fertilization on IWG and legume intercropping system. A legume service crop could fix atmospheric N_2 and supply N for the cereal intercrop, but a certain level of starter N is needed to overcome N stress until the nodules of legume are formed and capable for symbiotic N_2 fixation [34]. Increasing our understanding of how N fertilization impacts interspecific interaction and N use in early intercropping of IWG and WC is necessary to minimize the interspecific competition and maximize resource utilization in intercropping, thereby reducing the fertilizer inputs, minimizing environmental pollution, and optimizing agricultural productivity.

This study aimed to determine the effect of species-relative frequency and N fertilization on the competition for soil N sources, symbiotic N_2 fixation, dry matter yield, and intercropping advantage of IWG and WC intercropping systems during early growth. We hypothesized that (1) the symbiotic N_2 fixation will increase with the decrease in IWG-relative frequency, due to the decreased interspecific competition from IWG, (2) N fertilization will increase the interspecific competition at the advantages of IWG, and (3) higher N fertilizer levels will reduce intercropping advantages.

2. Materials and Methods

2.1. Greenhouse Experiment

The pot experiment was conducted in a greenhouse at China Agricultural University, Beijing, China, from 15 February to 4 July 2019. Supplemental light was supplied with high pressure sodium lamps (400 W, 100 μmol m^{-2} s^{-1}) to give 16 h light and 8 h dark periods each day. The temperature was 26 °C during the day and 20 °C during the night, and the air humidity was kept at 50% in the greenhouse. A loam soil was collected from the top 10 cm of a soil profile at the Shangzhuang Experimental Station (39° 59′ N, 116° 17′ E) of China Agricultural University. The chemical properties of the soil were: total N 537 mg kg^{-1}, nitrate N 11.0 mg kg^{-1}, ammonium N 2.08 mg kg^{-1}, total phosphorus 686 mg kg^{-1}, available phosphorus 16.2 mg kg^{-1}, total potassium 11.6 g kg^{-1}, available potassium 75.5 mg kg^{-1}, pH_{H20} 8.21, and soil organic matter 11.7 g kg^{-1}. Faba bean (*Vicia faba* L.) was the preceding crop in the field. Soil samples were sieved using a 2 mm sieve and homogenized. Pots with a diameter of 285 mm and a height of 265 mm (approx. 5 L) were filled with 10 kg soil and 4 L water added to each (70% water holding capacity).

The pot experiment followed a two-factor complete randomized design. The first factor was 4 levels of inorganic nitrogen fertilizer; N0, N1, N2, N3, corresponding to 0, 0.48, 0.96, and 1.44 g N pot^{-1}, which equaled approximately 0, 75, 150, and 225 kg N ha^{-1}, respectively. The second factor was 5 levels of species-relative frequency. In total, 16 plants per pot were planted according to a replacement series design, where intermediate wheatgrass (*Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey, Cycle 3 from The Land Institute, a non-profit organization, Salina, Kansas, USA) (IWG) and white clover (*Trifolium repens* L.) (WC) were grown at five mixtures as 100% IWG (all 16 plants IWG), 75% IWG (12 plants IWG, 4 plants WC), 50% IWG (8 plants IWG, 8 plants WC), 25% IWG (4 plants IWG, 12 plants WC), and 0% IWG (0 plants IWG, 16 plants WC). Each treatment combination was replicated three times. There were 60 pots of plants (4 × 5 × 3) in this experimental design. The ^{15}N-labeled ammonium nitrate ($^{15}NH_4{}^{15}NO_3$, 10.1% ^{15}N) was used as the nitrogen fertilizer applied to the ^{15}N-labeled treatments together with KCl. Two extra pots were supplied unlabeled N-fertilizer (ordinary KNO_3 and NH_4Cl) as a control to measure the background $\delta^{15}N$ value for the calculation of symbiotic N_2 fixation and apparent transfer of N. KCl was added to ^{15}N-labeled treatments for keeping the form of the ions in fertilizers applied to treatments were as same as that of controls. Nitrogen fertilizer application was split into three applications to ensure the success of ^{15}N isotope labeling, stabilize soil ^{15}N enrichment by regular additions, and improve synchrony of N supply and demand. ^{15}N-labeled fertilizer (N1, N2, N3) mother liquors were prepared using 6.84, 13.7, 20.5 g of $^{15}NH_4{}^{15}NO_3$ mixed with 6.37, 12.7, 19.1 g KCl and dissolved in 1 L distilled water, respectively. An aliquot of 66 mL mother liquor was diluted to 1 L and irrigated to each pot correspondingly to give 0, 0.16, 0.32, 0.48 g N pot^{-1} at each application. For unlabeled controls, ordinary N fertilizer (N1, N2, N3) mother liquors were prepared using 5.75, 11.5, 17.3 g KNO_3 mixed with 3.05, 6.09, 9.14 g NH_4Cl and dissolved according to above. In total, 0, 0.48, 0.96, 1.44 g N pot^{-1} was applied to N1, N2, N3 treatments after three applications of N fertilizer irrespective of ^{15}N labeled treatments or unlabeled controls. Seeds of IWG were provided by the Swedish University of Agricultural Sciences. The seeds of WC were pre-inoculated with rhizobia bacteria (*Rhizobium leguminosarum biovar trifolii*). The agronomic practices and treatments are described in Table 1.

Table 1. The description of agronomic practices and treatments.

Date	Agronomic Practices	Description
16 February	Sowing	Seeds of intermediate wheatgrass (IWG) and white clover (WC) were sown simultaneously.
From 25 February	Watering	500 mL water was irrigated to each pot weekly to keep soil moisture at 70% water holding capacity.
3 to 10 March	Thinning	Five species-relative frequencies were formed by thinning seedlings.
From 19 March	Watering	1 L water was irrigated once every four days to keep soil moisture at 70% water holding capacity.
2 April	First N fertilizer application	$^{15}NH_4{}^{15}NO_3$ (10.1% ^{15}N) and KCl were applied for ^{15}N-labeled treatments, and KNO_3 and NH4Cl were applied for controls.
6 April	Spraying pesticides	Pesticide thiosemicarbazide was sprayed on plants to control pest aphid.
28 April	Second N fertilizer application	$^{15}NH_4{}^{15}NO_3$ (10.1% ^{15}N) and KCl were applied for ^{15}N-labeled treatments, and KNO_3 and NH_4Cl were applied for controls.
4 May	Spraying pesticides	Pesticide avermectin was sprayed on plants to control pest red spiders.
From 8 May	Watering	2 L water was irrigated once every two days to keep soil moisture at 70% water holding capacity.
6 June	Third N fertilizer application	$^{15}NH_4{}^{15}NO_3$ (10.1% ^{15}N) and KCl were applied for ^{15}N-labeled treatments, and KNO_3 and NH_4Cl were applied for controls.

Table 1. *Cont.*

Date	Agronomic Practices	Description
16 June	Spraying pesticides	Pesticide bifenthrin was sprayed on plants to control pest pieris brassicae.
2 July	Harvest and sampling	Shoots and roots of IWG and WC and soil samples were collected.

2.2. Plant and Soil Analyses

2.2.1. Dry Matter Yield

The harvest was done at the full-bloom stage of WC and the heading stage of IWG. Shoots were cut at the soil level and separated into IWG and WC shoots. The soil was removed from the pots, and roots were sifted out of the soil by using a sieve (2 mm). The roots of IWG and WC were separated according to their different shapes, colors, and the presence of nodules, after washing in tap water. All shoots and roots samples were oven-dried at 60 °C for 72 h for the measurements of the shoot and root dry matter.

2.2.2. ^{15}N Abundance

Plant materials were ground to a fine powder by using two milling machines for the analyses of total nitrogen concentration and ^{15}N abundance. Plant samples were sent to the Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, for isotope ratio mass spectrometry analyses of nitrogen isotopes.

2.2.3. Soil Inorganic N and pH

After storage at −20 °C, 50 mL 1 mol L^{-1} KCl was added to 12 g of fresh soil in 100 mL plastic tubes and shaken for 30 min at 250 rpm. The soil inorganic N concentration of extracts was analyzed using a continuous flow mass spectrometer (SEAL AutoAnalyzer 3) by the UV-absorbance spectrophotometer method [35]. Soil water content was measured based on the gravimetric method for the calculation of soil inorganic N concentrations. Soil pH was measured using a pH meter on the filter extract of 10 g air dried soil extracted in 50 mL distilled water after shaking for 30 min at 275 rpm.

2.3. Nitrogen Acquisition

2.3.1. N_2 Fixation and N Transfer

The proportion of N derived from the atmosphere of WC shoot or root ($\%NA_{\mathrm{SHOOT\ or\ ROOT}}$, %) was calculated following the ^{15}N isotope dilution method [36,37] using Equation (1).

$$\%NA_{\mathrm{SHOOT\ or\ ROOT}} = (1 - [\mathrm{atom\%}\ ^{15}\mathrm{N\ excess}_{\mathrm{WC}}/\mathrm{atom\%}\ ^{15}\mathrm{N\ excess}_{\mathrm{IWG\ SOLE}}]) \times 100 \quad (1)$$

The term "atom% ^{15}N excess" reflects the ^{15}N enrichment above the background levels of unlabeled growth environments, i.e., the atom% ^{15}N excess is atom% ^{15}N of labeled samples (three replicates) minus the atom% ^{15}N of unlabeled controls (two replicates). Here, the atom% ^{15}N excess$_{\mathrm{WC}}$ indicates the atom% ^{15}N excess of the legume crop WC, and the atom% ^{15}N excess$_{\mathrm{IWGSOLE}}$ indicates the atom% ^{15}N excess of the non-leguminous IWG sole crop. The calculation of %NA was done for the shoots and roots of WC separately, as well as for intercrops and sole crops of WC under each N fertilizer rate. That is, the atom% ^{15}N excess of shoots and roots of WC in intercrops was used to calculate the %NA of shoots and roots of intercropped WC for each N fertilizer rate, and atom% ^{15}N excess of shoots and roots of WC sole crops was used to calculate the %NA of shoots and roots of sole cropped WC for each fertilizer level, while always the atom% ^{15}N excess of shoots and roots of sole cropped IWG was used as the non-fixing reference to calculate %NA at a given N level.

The amount of N fixed by WC shoot or root ($\mathrm{NFIX_{SHOOT\ or\ ROOT}}$, g $\mathrm{pot^{-1}}$) was determined using Equation (2) [36], where $\mathrm{Y_{WC}}$ is the dry matter yield of WC shoot or root, $\mathrm{\%N_{WC}}$ is the N concentration of WC shoot or root.

$$\mathrm{NFIX_{SHOOT\ or\ ROOT}} = \mathrm{Y_{WC}} \times \mathrm{\%N_{WC}}/100 \times \mathrm{\%NA_{SHOOT\ or\ ROOT}}/100 \quad (2)$$

The proportion of fixed N of WC whole plant ($\mathrm{\%NA_{WC\ TOTAL}}$, %) was calculated using Equation (3), where $\mathrm{NFIX_{SHOOT}}$ and $\mathrm{NFIX_{ROOT}}$ indicate the amount of N fixed by WC shoot and root, respectively, while $\mathrm{N_{SHOOT}}$ and $\mathrm{N_{ROOT}}$ indicate the N accumulated in WC shoots and roots, respectively. Thereafter, the accumulation of N in WC was calculated by multiplying the N concentration of WC by the dry matter of WC.

$$\mathrm{\%NA_{WC\ TOTAL}} = (\mathrm{NFIX_{SHOOT}} + \mathrm{NFIX_{ROOT}})/(\mathrm{N_{SHOOT}} + \mathrm{N_{ROOT}}) \times 100 \quad (3)$$

The percentage of N in IWG intercrops apparently transferred from WC intercrops was calculated by comparing ^{15}N enrichment in IWG mixed intercrops versus IWG sole crop at a given N level, following the ^{15}N isotope dilution method [36,37]. The percentage of N apparently transferred to IWG shoot and root ($\mathrm{\%NT_{SHOOT\ or\ ROOT}}$, %) was calculated separately using Equation (4) [37], where atom% ^{15}N $\mathrm{excess_{IWGMIX}}$ indicates the atom% ^{15}N excess of IWG mixed intercrops, and atom% ^{15}N $\mathrm{excess_{IWGSOLE}}$ indicates the atom% ^{15}N excess of IWG sole at each N fertilizer level.

$$\mathrm{\%NT_{SHOOT\ or\ ROOT}} = (1 - [\mathrm{atom\%\ ^{15}N\ excess_{\ IWG\ MIX}}/\mathrm{atom\%\ ^{15}N\ excess_{\ IWG\ SOLE}}]) \times 100 \quad (4)$$

Then, the amount of N apparently transferred to IWG shoot or root ($\mathrm{NT_{SHOOT\ or\ ROOT}}$, g $\mathrm{pot^{-1}}$) was determined for each IWG intercrop under each relative frequency and N fertilizer rate using Equation (5) [37], where $\mathrm{Y_{IWG}}$ is the dry matter yield of IWG shoot or root, and $\mathrm{\%N_{IWG}}$ is the N concentration of IWG shoot or root at a given IWG frequency and given N fertilizer rate.

$$\mathrm{NT_{SHOOT\ or\ ROOT}} = \mathrm{Y_{IWG}} \times \mathrm{\%N_{IWG}}/100 \times \mathrm{\%NT_{SHOOT\ or\ ROOT}}/100 \quad (5)$$

The percentage of N apparently transferred to IWG whole plant ($\mathrm{\%NT_{IWG\ TOTAL}}$, %) was calculated using Equation (6), where $\mathrm{NT_{SHOOT}}$ represents the amount of N transferred to IWG shoot, and $\mathrm{NT_{ROOT}}$ represents the amount of N transferred to IWG root, $\mathrm{N_{SHOOT}}$ and $\mathrm{N_{ROOT}}$ represent the amount of N accumulation of IWG shoot and root, respectively.

$$\mathrm{\%NT_{IWG\ TOTAL}} = (\mathrm{NT_{SHOOT}} + \mathrm{NT_{ROOT}})/(\mathrm{N_{SHOOT}} + \mathrm{N_{ROOT}}) \times 100 \quad (6)$$

2.3.2. N Derived from Fertilizer and Soil

The proportion of N derived from the fertilizer (%NF) was estimated by comparing ^{15}N enrichment in the plant (IWG and WC) versus ^{15}N enrichment in the labeled fertilizer at each IWG-relative frequency and N fertilizer rate using Equation (7) [34,38]. The atom% ^{15}N excess of IWG was used for calculating the %NF of IWG, and atom% ^{15}N excess of WC was used for calculating the %NF of WC. The same atom% ^{15}N excess of N fertilizer was used for the calculation of %NF in IWG or WC at a given N fertilizer rate.

$$\mathrm{\%NF} = (\mathrm{atom\%\ ^{15}N\ excess_{IWG\ or\ WC}}/\mathrm{atom\%\ ^{15}N\ excess_{FERTILIZER}}) \times 100 \quad (7)$$

The proportion of N derived from the unlabeled soil (%NS) was calculated with the assumption that N accumulated in WC and IWG arise from fertilizer and soil in both cases, while also from the atmosphere for WC and from transfer in IWG [39,40] (Equation (8) for WC and Equation (9) for IWG). The %NF represents the proportion of N derived from fertilizer, %NS the proportion of N derived from soil, %NA the proportion of N derived from the atmosphere, and %NT the proportion of N transferred from WC to IWG.

$$\mathrm{\%NF} + \mathrm{\%NS} + \mathrm{\%NA} = 100\% \quad (8)$$

$$\%NF + \%NS + \%NT = 100\% \tag{9}$$

The amount of N derived from fertilizer in plant shoot and root were calculated by multiplying the %NF of the plant shoot or root by the dry matter of shoot or root, which were summed up to give the amount of N derived from fertilizer in the whole plant. The %NF of the whole plant was calculated by dividing the amount of N derived from the fertilizer of whole plants by the N accumulation of whole plants and multiplying by 100. The same method was used to calculate the %NS of the whole plant.

Fertilizer N recovery (%) by the crop was calculated for each treatment by the equation presented by IAEA [38] and Jørgensen et al. [36] (Equation (10)). The %NF, total $N_{IWG\ or\ WC}$, and total $N_{FERTILIZER}$ are derived from the calculations above.

$$\text{Recovery} = (\%NF \times \text{total } N_{IWG\ or\ WC} / \text{total } N_{FERTILIZER}) \times 100 \tag{10}$$

The same amount of N fertilizer was used for calculating both IWG and WC fertilizer N recoveries. Then, the total recovery for the whole cropping systems was calculated as the sum of N recoveries of IWG and WC.

2.4. Intercropping Advantages and Interspecific Interactions

2.4.1. Relative Yield Total

The relative advantage of mixed intercropping compared to sole cropping was estimated by the relative yield total (RYT) [27] (Equation (11)), where $Y_{IWG\ MIX}$ and $Y_{WC\ MIX}$ indicate the dry matter yields of IWG and WC mixed intercrops per pot, $Y_{IWG\ SOLE}$ and $Y_{WC\ SOLE}$ indicate the mean of dry matter yields of five pots with IWG and WC sole crops under the same N fertilizer level.

$$RYT = (Y_{IWG\ MIX}/Y_{IWG\ SOLE}) + (Y_{WC\ MIX}/Y_{WC\ SOLE}) \tag{11}$$

An RYT larger than one indicates an advantage for intercropping compared to sole cropping. An RYT less than one indicates an advantage for sole cropping, while an RYT of one indicates no advantages from mixed intercropping compared to sole cropping.

2.4.2. Competitive Ratio

The competitive ratio of IWG (CR_{IWG}) was used as an indicator to evaluate the competitive ability of IWG relative to WC, and the CR_{WC} was used to evaluate the competitive ability of WC relative to IWG. The competitive ratio represents the ratio of individual RYTs of the two component crops and takes into account the proportion of the crops in which they are initially sown [41] (Equations (12) and (13)).

$$CR_{IWG} = (Y_{IWG\ MIX}/Y_{IWG\ SOLE} \times IRF)/(Y_{WC\ MIX}/Y_{WC\ SOLE} \times [1\text{-}IRF]) \tag{12}$$

$$CR_{WC} = 1/CR_{IWG} \tag{13}$$

The $Y_{IWG\ MIX}$ and $Y_{WC\ MIX}$ represent the dry matter yields of IWG and WC mixed intercrops per pot, $Y_{IWG\ SOLE}$ and $Y_{WC\ SOLE}$ represent the dry matter yields of IWG and WC sole crops per pot. IRF is the IWG-relative frequency, which equals the initial sown proportion of IWG intercrops, and 1-IRF is the WC-relative frequency, which equals the initial sown proportion of WC intercrops. When CR_{IWG} is greater than one, the competitive ability of IWG is higher than WC in mixed intercrops. Contrarily, when the CR_{WC} is greater than one, the competitive ability of WC is higher than IWG.

2.5. Statistical Analyses

The main effects of N fertilizer and species-relative frequency, and the N fertilizer × species-relative frequency interaction were assessed using analysis of variance (two-way ANOVA), performed by the general linear model (GLM) in IBM SPSS statistics 23.0. When the effect of the treatments was found to be significant (*F*-tests, p-value < 0.05), means were compared using Tukey's HSD test at α = 0.05. All the measured variables fulfilled the assumptions of normal distribution and homogenous variances. Three replicates were used in the analysis of variance and calculation of means and standard error for all responsible variables.

3. Results

3.1. IWG and WC Dry Matter Yield

The shoot and root dry matter productions of most IWG were not significantly influenced by a decrease in IWG-relative frequency within each N fertilizer level, except for the 25% IWG, which has lower yields than IWG sole crop at N2 and N3 (Figure 1). Within a specific IWG-relative frequency, the IWG shoot dry matter increased with increased N supply, reaching apparently a maximum at the N3 nitrogen fertilizer level. The IWG root dry matter was higher at the N1 fertilization level compared to the N0, but additional N did not increase root dry matter further. The shoot and root dry matters of WC increased as the level of IWG-relative frequency decreased, reaching a maximum in the sole crop WC, within each N fertilizer level (Figure 1). Nitrogen fertilization did not significantly influence the WC shoot and root dry matter productions. The shoot and root dry matters of WC were always lower than the dry matter of IWG regardless of species-relative frequencies and N fertilizer levels in intercrops.

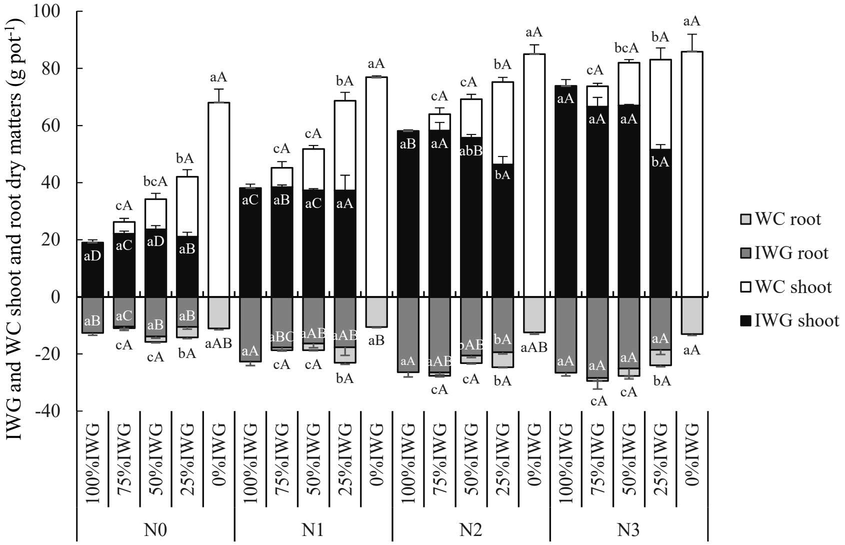


Figure 1. Shoot and root dry matter of intermediate wheatgrass (IWG) and white clover (WC) per pot under four N fertilizer levels (N0, N1, N2, and N3) and five IWG-relative frequencies (IRF) (100% IWG, 75% IWG, 50% IWG, 25% IWG, 0% IWG). The absolute values of numbers on the negative side of the *Y*-axis are the root dry matter of IWG and WC. Different lower-case letters indicate significant differences at $p < 0.05$ among IRF under the same N level, and different upper-case letters indicate significant differences at $p < 0.05$ among N levels under the same IRF (Tukey's post hoc test).

The total dry matters of IWG and WC intercrops at 50 and 25% IWG were higher than that of the IWG sole crop but lower than WC sole crop at N0 (Table 2). The total dry matter of IWG and WC intercrop at 25% IWG was higher than IWG sole crop and similar to WC sole crop at N1 and N2. There was no significant difference between intercrops and sole crops at N3. Within a specific IWG-relative frequency, the total dry matter of IWG and WC intercrops increased with the increase in the N fertilizer level. The IWG root/shoot ratio was significantly reduced with increasing N fertilizer level at 100% IWG and 50% IWG, while the WC root/shoot ratio at 50% IWG was significantly higher than the WC sole crop at N0 fertilizer level.

Table 2. The total dry matter yields per pot, root/shoot ratio of intermediate wheatgrass (IWG) and white clover (WC), relative yield total (RYT), and the competitive ratio of IWG (CR_{IWG}) and WC (CR_{WC}) under five relative frequencies of IWG (IRF) and four N fertilizer levels (N). Data are presented as mean ± standard error (*n* = 5). F-statistics and significance from ANOVA are reported below the treatment means.

N	IRF	Total Yields (g Pot^{-1})	IWG Root/Shoot	WC Root/Shoot	RYT	CR_{IWG}	CR_{WC}
N0	100%IWG	32.5 ± 1.13cD	0.63 ± 0.00A				
	75%IWG	37.6 ± 1.15cD	0.51 ± 0.11A	0.15 ± 0.01b	1.05 ± 0.04aA	7.04 ± 2.02A	0.17 ± 0.05a
	50%IWG	51.0 ± 2.59bD	0.57 ± 0.03A	0.18 ± 0.01ab	1.31 ± 0.03aA	8.16 ± 1.13A	0.13 ± 0.02a
	25%IWG	55.4 ± 1.99bB	0.51 ± 0.07A	0.19 ± 0.01a	1.30 ± 0.01aA	9.43 ± 1.48A	0.11 ± 0.02a
	0%IWG	79.0 ± 5.23aA		0.15 ± 0.01b			
N1	100%IWG	60.8 ± 2.34cC	0.60 ± 0.03A				
	75%IWG	63.8 ± 2.76cC	0.46 ± 0.04A	0.15 ± 0.02a	1.07 ± 0.03aA	5.12 ± 2.36A	0.28 ± 0.09a
	50%IWG	70.5 ± 0.85bcC	0.44 ± 0.03AB	0.16 ± 0.01a	1.13 ± 0.02aB	4.84 ± 0.58A	0.21 ± 0.03a
	25%IWG	91.8 ± 8.82aA	0.47 ± 0.02A	0.17 ± 0.00a	1.39 ± 0.15aA	6.77 ± 1.08A	0.16 ± 0.03a
	0%IWG	87.5 ± 0.57abA		0.14 ± 0.00a			
N2	100%IWG	84.5 ± 1.37bB	0.45 ± 0.03B				
	75%IWG	91.6 ± 1.70abB	0.46 ± 0.01A	0.21 ± 0.01a	1.10 ± 0.02aA	6.47 ± 2.57A	0.22 ± 0.09a
	50%IWG	92.4 ± 2.05abB	0.37 ± 0.01B	0.20 ± 0.02a	1.10 ± 0.02aB	5.56 ± 0.74A	0.19 ± 0.02a
	25%IWG	99.9 ± 3.70aA	0.42 ± 0.03A	0.18 ± 0.01a	1.16 ± 0.04aA	6.61 ± 0.41A	0.15 ± 0.01a
	0%IWG	97.4 ± 2.85aA		0.15 ± 0.01a			
N3	100%IWG	100 ± 1.34aA	0.36 ± 0.03B				
	75%IWG	103 ± 3.06aA	0.43 ± 0.08A	0.15 ± 0.03a	1.03 ± 0.03aA	4.00 ± 0.59A	0.26 ± 0.04a
	50%IWG	110 ± 3.69aA	0.38 ± 0.06B	0.17 ± 0.01a	1.10 ± 0.04aB	5.23 ± 0.39A	0.19 ± 0.01a
	25%IWG	107 ± 4.36aA	0.36 ± 0.04A	0.18 ± 0.02a	1.07 ± 0.04aA	5.83 ± 0.79A	0.18 ± 0.02a
	0%IWG	98.9 ± 6.54aA		0.15 ± 0.01a			
				F-statistic			
Source of variation							
N		213 ***	10.5 ***	2.72	5.39 **	3.03 *	2.06
IRF		26.4 ***	2.16	5.04 **	10.2 **	1.33	3.60 *
N*IRF		6.77 ***	1.06	1.04	2.99 *	0.20	0.11

Notes: Different lower-case letters indicate significant differences at $p < 0.05$ among IRF under the same N level, and different upper-case letters indicate significant differences at $p < 0.05$ among N levels under the same IRF (Tukey's post hoc test). IRF means the species-relative frequency of IWG. Asterisks indicate significant differences, where * indicates $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3.2. Intercropping Advantages and Interspecific Interactions

The relative yield total (RYT) did not differ among the IWG-relative frequencies under all N fertilizer conditions (Table 2). Nitrogen fertilization did not have any effect on RYT at 75% IWG and 25% IWG. However, at 50% IWG, RYT was higher under the N0 fertilization level than under the N1, N2, and N3 levels. The competitive ratio of IWG (CR_{IWG}) was larger than one under all treatments, while the competitive ratio of WC (CR_{WC}) was less than one.

3.3. The Proportion of N Derived from Different N Sources

Both the proportion of N derived from soil (%NS) and fertilizer (%NF) of IWG had a tendency to decrease with the decrease in IWG-relative frequency at N1 and N2 fertilizer levels and remained unchanged at N3 (Figure 2). The proportion of apparent transfer N (%NT) was unaffected by the decrease in IWG-relative frequency at all N fertilizer levels. Within a specific IWG-relative frequency, the %NS decreased with the increase in N fertilizer rates, while the %NF increased, and %NT remained unchanged.

Both %NS and %NF of WC intercrops are lower than WC sole crops at all N fertilizer levels (Figure 3). Conversely, the proportions of N derived from N_2 fixation (%NA) in WC intercrops are higher than in WC sole crops at all N fertilizer levels. There were no significant differences among different WC intercrops (75% IWG, 50% IWG, and 25% IWG), either of %NA, %NF, or %NS. Within a specific IWG-relative frequency, the %NS of WC remained constant with the increase in N fertilizer levels, while the %NF increased, and %NA decreased sharply.

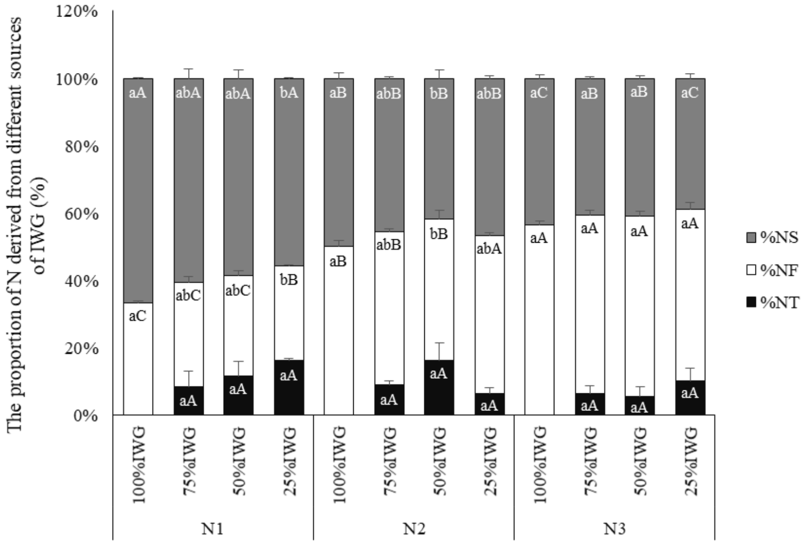


Figure 2. The proportion of N derived from the soil (%NS), fertilizer (%NF), and apparently transferred from white clover (%NT) in intermediate wheatgrass (IWG) whole plant under three N fertilizer levels (N1, N2, and N3) and four IWG-relative frequencies (IRF) (100% IWG, 75% IWG, 50% IWG, 25% IWG). Different lower-case letters indicate significant differences at $p < 0.05$ among IRF under the same N level, and different upper-case letters indicate significant differences at $p < 0.05$ among N levels under the same IRF (Tukey's post hoc test).

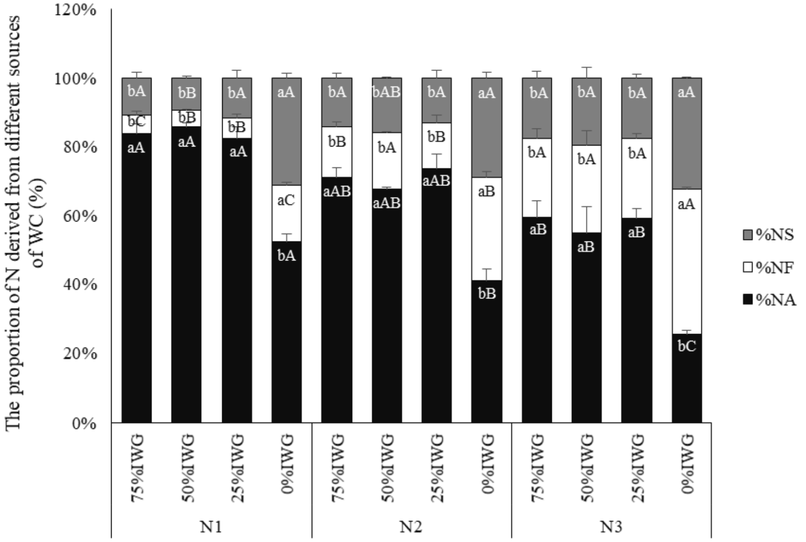


Figure 3. The proportion of N derived from the soil (%NS), fertilizer (%NF), and atmosphere (%NA) in white clover (WC) whole plant under three N fertilizer levels (N1, N2, and N3) and four IWG-relative frequencies (IRF) (75% IWG, 50% IWG, 25% IWG, and 0% IWG). Different lower-case letters indicate significant differences at $p < 0.05$ among IRF under the same N level, and different upper-case letters indicate significant differences at $p < 0.05$ among N levels under the same IRF (Tukey's post hoc test).

3.4. Nitrogen Accumulation and Transfer

3.4.1. Nitrogen Accumulation

The total N accumulations of IWG and WC intercrops at 50 and 25% IWG were higher than that of the IWG sole crop but lower than WC sole crop at N0 (Table 3). The total N accumulations of intercrops at 50 and 25% IWG were higher than that of the IWG sole crop, and only the N accumulation at 25% IWG was similar to WC sole crop at N1 and N2. The total N accumulation of intercrop at 25% IWG was higher than that of the IWG sole crop and similar to WC sole crop at N3. The total N accumulation of intercrops tended to increase with the decrease in IWG-relative frequency from 75% IWG to 25% IWG. Within a specific species-relative frequency, the total N accumulations increased with the increase in the N fertilizer level except for the total N accumulation of WC sole crop. Nitrogen accumulation of IWG increased with the increase in N fertilizer level under the same IWG-relative frequency, reaching a maximum at the highest N fertilizer level N3. Nitrogen accumulation of WC increased with the decrease in IWG-relative frequency under all fertilizer levels, reaching a maximum at sole crops (0% IWG).

Table 3. The amount of total N accumulation per pot (Total N), N accumulation of intermediate wheatgrass (IWG N) and white clover whole plants (WC N), N_2 fixation of WC, and apparent transfer of N to IWG under five relative frequencies of IWG (IRF) and four N fertilizer levels (N). Data are presented as mean ± standard error. F-statistics and significance from ANOVA are reported below the treatment means.

N	IRF	Total N (g Pot^{-1})	IWG N (g Pot^{-1})	WC N (g Pot^{-1})	N_2 Fixation (g Pot^{-1})	N Transfer (g Pot^{-1})
N0	100%IWG	0.37 ± 0.03dD	0.37 ± 0.03D			
	75%IWG	0.47 ± 0.06cdC	0.36 ± 0.02C	0.09 ± 0.03cA	ND	ND
	50%IWG	0.72 ± 0.05bcD	0.46 ± 0.01D	0.24 ± 0.03bcA	ND	ND
	25%IWG	0.91 ± 0.04bC	0.42 ± 0.01C	0.49 ± 0.05bA	ND	ND
	0%IWG	1.56 ± 0.10aA		1.56 ± 0.10aA	ND	ND
N1	100%IWG	0.70 ± 0.02cC	0.70 ± 0.02C			
	75%IWG	0.80 ± 0.05bcB	0.65 ± 0.02B	0.15 ± 0.05dA	0.12 ± 0.04cA	0.06 ± 0.03
	50%IWG	1.03 ± 0.02bC	0.70 ± 0.01C	0.33 ± 0.03cA	0.28 ± 0.02cA	0.08 ± 0.03
	25%IWG	1.43 ± 0.12aB	0.74 ± 0.10B	0.69 ± 0.05bA	0.57 ± 0.05bA	0.12 ± 0.01
	0%IWG	1.71 ± 0.01aA		1.71 ± 0.01aA	0.89 ± 0.04aA	
N2	100%IWG	1.08 ± 0.03cB	1.08 ± 0.03B			
	75%IWG	1.33 ± 0.02bcA	1.20 ± 0.06A	0.13 ± 0.05cA	0.09 ± 0.04cA	0.11 ± 0.02
	50%IWG	1.35 ± 0.04bB	1.05 ± 0.05B	0.30 ± 0.03cA	0.20 ± 0.02cA	0.18 ± 0.06
	25%IWG	1.69 ± 0.08aAB	1.04 ± 0.05A	0.64 ± 0.04bA	0.48 ± 0.05bA	0.07 ± 0.02
	0%IWG	1.81 ± 0.08aA		1.81 ± 0.08aA	0.75 ± 0.09aA	
N3	100%IWG	1.40 ± 0.03bA	1.40 ± 0.03A			
	75%IWG	1.47 ± 0.04bA	1.33 ± 0.05A	0.14 ± 0.02cA	0.08 ± 0.00bA	0.08 ± 0.03
	50%IWG	1.65 ± 0.05abA	1.34 ± 0.04A	0.31 ± 0.02cA	0.17 ± 0.03bA	0.07 ± 0.04
	25%IWG	1.84 ± 0.07aA	1.17 ± 0.03A	0.67 ± 0.08bA	0.40 ± 0.06aA	0.12 ± 0.04
	0%IWG	1.84 ± 0.08aA		1.84 ± 0.08aA	0.47 ± 0.04aB	
			F-statistic			
Source of variation						
N		192 ***	369 ***	5.91 **	16.6 ***	0.78
IRF		133 ***	1.03	722 ***	106 ***	0.50
N*IRF		6.40 ***	3.12 **	1.00	3.65 *	1.75

Notes: Different lower-case letters indicate significant differences at $p < 0.05$ among IRF under the same N level, and different upper-case letters indicate significant differences at $p < 0.05$ among N levels under the same IRF (Tukey's post hoc test). IRF means the species-relative frequency of IWG. ND means not determined. Asterisks indicate significant differences, where * indicate $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3.4.2. N_2 Fixation and Apparent Transfer of N

The amount of N_2 fixed by WC sole crop was higher than WC intercrops at all N fertilizer levels, and the N_2 fixation of WC intercrops tended to increase with the decrease in IWG-relative frequency, with no differences found among 75% IWG and 50% IWG

(Table 3). Within a specific relative frequency, only N_2 fixation of WC sole crop was lower at N3 than N1 and N2 fertilizer levels. The amount of apparent N transfer from WC to IWG was unaffected by species-relative frequency or N fertilizer.

3.5. Soil Inorganic N Concentration after Harvest

The total fertilizer N recovery is affected by N fertilizer levels (Table 4). The total fertilizer N recovery in the 75% IWG treatment was higher at the N2 fertilizer level than N1. The fertilizer N recovery of IWG decreased with the decrease in IWG-relative frequency at N2 and N3 fertilizer levels. Within a specific IWG-relative frequency, the N recovery of 75% IWG was higher at N2 than the N1 fertilizer level. The fertilizer N recovery of WC was affected by species-relative frequency; the N recovery of WC increased with the decrease in IWG-relative frequency. The soil mineral N concentration tended to increase with the increase in N fertilizer level under treatments of 100% IWG, 50% IWG, and 0% IWG. No significant differences were detected among IWG-relative frequencies irrespective of the N fertilizer level. Soil pH of IWG and WC intercrops at 50 and 25% IWG were lower than IWG and WC sole crops at N0. Soil pH of WC sole crop was higher than mixed intercrops and IWG sole crop at N1 fertilizer level, with no difference between the mixed intercrops and the IWG sole crop. Within a specific IWG-relative frequency, soil pH decreased with the increase in N fertilizer levels at 100% IWG and 75% IWG.

Table 4. The total fertilizer N recovery (Recovery total), N recovery of intermediate wheatgrass (Recovery IWG) and white clover (Recovery WC), the concentration of soil mineral N, and pH value under five relative frequencies of IWG (IRF) and four N fertilizer levels (N). Data are presented as mean ± standard error. F-statistics and significance from ANOVA are reported below the treatment means.

N	IRF	Recovery Total (%)	Recovery IWG (%)	Recovery WC (%)	Soil Mineral N (mg kg^{-1})	pH
N0	100%IWG				3.14 ± 0.22aB	8.16 ± 0.02aA
	75%IWG				3.44 ± 0.24aA	8.10 ± 0.04abA
	50%IWG				2.86 ± 0.32aB	8.00 ± 0.05bcA
	25%IWG				2.98 ± 0.18aA	7.93 ± 0.02cA
	0%IWG				2.64 ± 0.09aB	8.21 ± 0.01aA
N1	100%IWG	49.2 ± 1.66A	49.2 ± 1.66aA		4.34 ± 0.57aAB	7.88 ± 0.04bB
	75%IWG	43.5 ± 2.15B	42.0 ± 1.83aB	1.50 ± 0.33b	3.58 ± 0.03aA	7.86 ± 0.03bBC
	50%IWG	47.0 ± 2.39A	43.7 ± 2.85aA	3.34 ± 0.51b	3.28 ± 0.44aB	7.95 ± 0.01bA
	25%IWG	52.4 ± 5.20A	43.8 ± 6.47aA	8.66 ± 2.12b	3.19 ± 0.20aA	7.96 ± 0.07bA
	0%IWG	58.4 ± 2.73A		58.4 ± 2.73a	3.51 ± 0.42aAB	8.39 ± 0.03aA
N2	100%IWG	56.6 ± 0.27A	56.6 ± 0.27aA		5.64 ± 0.87aA	7.78 ± 0.02aB
	75%IWG	59.2 ± 1.90A	57.4 ± 2.35aA	1.82 ± 0.51d	4.01 ± 0.43aA	7.73 ± 0.05aC
	50%IWG	51.3 ± 2.70A	46.2 ± 2.34bA	5.08 ± 0.46c	4.24 ± 0.28aAB	7.78 ± 0.04aA
	25%IWG	60.0 ± 1.08A	51.2 ± 1.68abA	8.85 ± 0.90b	4.63 ± 0.40aA	7.93 ± 0.16aA
	0%IWG	56.4 ± 0.71A		56.4 ± 0.71a	3.84 ± 0.22aAB	7.94 ± 0.23aA
N3	100%IWG	55.4 ± 2.52A	55.4 ± 2.52aA		5.64 ± 0.14aA	7.92 ± 0.08aB
	75%IWG	51.5 ± 2.63AB	49.1 ± 3.10abAB	2.36 ± 0.50c	4.76 ± 0.40aA	7.92 ± 0.03aAB
	50%IWG	56.5 ± 2.25A	51.0 ± 3.06abA	5.43 ± 0.81c	5.16 ± 0.27aA	7.91 ± 0.10aA
	25%IWG	52.0 ± 1.49A	41.5 ± 2.09bA	10.6 ± 0.71b	4.75 ± 0.77aA	8.17 ± 0.02aA
	0%IWG	54.1 ± 1.86A		54.1 ± 1.86a	4.21 ± 0.29aA	8.12 ± 0.17aA
			F-statistic			
Source of variation						
N		9.75 **	8.10 **	0.01	23.3 ***	7.61 ***
IRF		2.34	4.73 *	1252 ***	4.47 **	6.57 ***
N*IRF		3.10 *	1.90	1.50	0.82	1.80

Notes: Different lower-case letters indicate significant differences at $p < 0.05$ among IRF under the same N level, and different upper-case letters indicate significant differences at $p < 0.05$ among N levels under the same IRF (Tukey's post hoc test). IRF means the species-relative frequency of IWG. Asterisks indicate significant differences, where * indicate $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

4. Discussion

4.1. Dry Matter Production, Complementary Interactions, and RYT

All values of RYT were larger than one in our study indicated that intercropping of IWG and WC has yield advantages under all species-relative frequencies. For most IWG intercrops, the shoot and root dry matter were similar to that of IWG sole crops, although the relative frequencies of IWG in intercropping were lower than in sole cropping. The 25% IWG intercrops produced the same yields as 100% IWG at N0 and N1 indicating that IWG has a high relative fitness and maintained a high total dry matter production even at low relative frequencies. This result supports the findings reported by Hunter et al. [42] that lower planting density in terms of winder row spacing tended to increase the mean grain yield of IWG. Although in a mixed intercropping system, the dry matter of IWG was not negatively affected by the interspecific competition from WC intercrops. As the result of the competitive ratio showed ($CR_{IWG} > 1$, $CR_{WC} < 1$), the competitive ability of WC was always much lower than that of IWG, and it has not been affected by species-relative frequency or N fertilizer rates. The results of N accumulation and fertilizer N recovery of IWG intercrops also showed that a comparable amount of N with that in IWG sole crop was accumulated in IWG intercrops despite low IWG-relative frequency. Our results suggest the improvement of dry matter yield and N content of IWG should not rely on overcrowding in sole cropping but the exploitation of complementarity and beneficial interactions between IWG and WC intercrops.

The shoot and root dry matters of WC intercrops were lower than WC sole crops but increased with the decrease in IWG-relative frequency within a specific N fertilizer rate, resulting in an upward tendency of system total dry matter of IWG and WC intercrops. The intercropping advantages of IWG and WC (RYT > 1) in this study were credited to the complementary use of N sources and N transfer from WC to IWG. Under N1 fertilizer condition, WC intercrops fulfilled their N requirement (%NA > 80%) by symbiotic N_2 fixation and saved the soil N for IWG intercrops (%NS > 50%), and an average of 12.3% N in IWG intercrops was transferred from WC intercrops. Moreover, the %NA of WC increased from 52.4% in sole crop to an average of 84.0% when intercropped with IWG at N1 due to the high competition of IWG for soil mineral N. These results once again confirmed the widespread theories about the mechanism of intercropping advantages: the complementary use of different N sources by cereal and legume intercrops in low input cropping systems [43], legumes facilitate the growth of associated cereals by transferring N [44], and cereals stimulate N_2 fixation of legumes through competition for mineral N in the rhizosphere [27].

4.2. Use of Different Nitrogen Sources

Nitrogen accumulations followed the pattern of dry matter yields. The N accumulation of IWG increased with the increase in N fertilizer rates, N accumulation of WC increased with the decrease in IWG-relative frequency, and total N accumulation was affected by the positive interaction of N fertilizer and species-relative frequency. The highest total N accumulation of IWG and WC intercrops existed in 25% IWG with the N3 fertilizer level. In IWG and WC intercropping, IWG intercrops recovered a more than proportional share of fertilizer N sources (more than 40%) in intercropping due to the highly competitive ability, while WC recovered less than 11% of the fertilizer recovery. A similar result reported by Jensen [27] in barley and pea intercrops that the higher competitive ability of barley resulted in the recovery of fertilizer N in the pea to be less than 10% of the total fertilizer N recovery. The highly competitive ability of IWG for fertilizer N forced WC intercrops more relying on the N derived from the atmosphere. We detected that an average of 70.9% of N in all WC intercrops derived from air, only an average of 14.7% derived from fertilizer, and an average of 14.4% from soil under three N fertilizer levels. However, for WC sole crops the proportion of N derived from the air was only an average of 39.7% under three N fertilizer levels, indicating that intercropping with IWG enhanced the proportion of N derived from the atmosphere in WC intercrops, correspondingly reduced the N derived from soil and fertilizer. Different species-relative frequency did not affect the proportion of N derived from air, but N fertilizer application inhibited symbiotic N_2 fixation of WC, meanwhile increasing the proportion of N absorbed from fertilizer. The proportion of N derived from the atmosphere in WC intercrop decreased from an average of 84.0 to 57.9% with the increase in N fertilizer level from N1 to N3, and the proportion of N derived from the fertilizer increased from 5.45 to 23.9%. This was consistent with results from Ledgard and Steele [45] who reported that if soil inorganic N was abundant, clover took up relatively more soil N and the proportion of N derived from the atmosphere decreased.

For all IWG intercrops, N came mainly from fertilizer (an average of 42.5%) and soil (an average of 47.6%), only a small proportion, about 10.1% on average, came from apparent N transfer from WC under three N fertilizer levels. The result of measured apparent transferred N varies between different crop stages, measurements, and environmental conditions. Høgh-Jensen and Schjoerring [46] found that the average amount of N transferred from clover to ryegrass was equivalent to 3, 16, and 31% of the N accumulated in ryegrass in the first, second, and third production year. In a split root experiment, Jensen [27] found that barley obtained up to 19% of its N from intercropped pea when grown in association for 70 days in a soil with a low inorganic N content. Values ranging from 6 to 80% of total N in the grass have been published for N transfer from the legume to the associated grass [47]. Transfer of N from WC to IWG can occur via decomposition of legume root tissues and uptake of the released N by cereal, exudation of soluble N compounds by legumes and uptake by cereal, and transfer of N mediated by plant-associated mycorrhizae [37,48,49]. The transfer of N is mostly long term, as suggested by Jørgensen et al. [36]. In this study, 10.1% N of IWG transferred from WC after 136 days of growth, the potential for transfer is expected to be much higher on a longer time scale.

4.3. The Role of a White Clover Service Crop on Future N Supply

Intercropping of cereals and legume service crops is a good strategy to improve N supply and reduce the input of new N fertilizer, with the benefit of N_2 fixation and potential transfer of N. In our study, the white clover provided sufficient N to the IWG intercrop to achieve an average of 33.9 g pot^{-1} shoot and root total dry matter without N fertilization. White clover as a service crop can be a relevant contributor to IWG N nutrition and better growth. The amount of N_2 fixed was high due to the high %NA, even though the dry matter yields of WC were relatively low. The amount of N_2 fixed was correlated with the dry matter yield of WC, as observed in other investigations in clover and ryegrass mixtures [50], indicating that optimum growth conditions could contribute to high dry matter production of WC and further enhance the amount of N fixed. White clover was a weak competitor for inorganic N in intercropping of IWG and WC due to the ability of symbiotic N_2 fixation. White clover intercrop was also a weak competitor for light due to short height and shading from IWG at all relative frequency, as Kendall and Stringer [51] reported that the relative growth rates of clover plants decreased rapidly in response to shading. Our previous study [14] showed that alfalfa was very aggressive when intercropped with IWG. We suggest that white clover is a more suitable companion leguminous intercrop for IWG as compared to alfalfa. The intercrops of 75% WC with 25% IWG (3:1) is an optimum combination with relatively low interspecific competition, high amount N fixed, and high RYT in this study.

Furthermore, when we calculated how much N could be fixed per g of WC, we found that 1 g WC dry matter contributed an average of 11.8×10^{-3} g fixed N to the intercropping systems, which was equal to 11.8 kg N t^{-1} WC dry matter. This value was slightly higher than the reports from Hayes et al. [11] of subterranean clover (<10 kg N t^{-1} dry matter), probably because N fixed by WC roots was also included in the N_2 fixation in our study. When we compared the N accumulation of IWG intercrops at N0 with N accumulation of IWG sole crops at N1, we found that despite the low species-relative frequency, the IWG intercrops achieved comparable N accumulation (from 0.36 to 0.46 g pot^{-1}) at N0 to the IWG sole crop (0.70 g pot^{-1}) fertilized with 75 kg N ha^{-1}, suggesting that the

WC service crop can supply enough N for IWG under appropriate soil N fertility and species-relative frequencies.

5. Conclusions

This study showed that IWG and WC intercrops have the potential to improve the use efficiency of N source and land productivity due to competitive, facilitative interactions, complementary use of soil mineral N and atmospheric N_2, and N transfer from WC to IWG. The intercrops of IWG, which have a highly competitive ability for N, acquired a much larger proportion of soil and fertilizer N, consequently forcing WC intercrops more relying on the N derived from the atmosphere. Decreasing IWG-relative frequency from 75 to 25% did not affect the %NA, %NT, %NF, %NS, RYT, dry matter, and N accumulation of IWG, while increased dry matter and N accumulation of WC, resulting in the increases in amounts of N_2 fixed, total dry matter and N accumulation in IWG and WC mixed intercropping. The incremental levels of N fertilizers increased %NF of both WC and IWG, resulting in decreased %NA of WC and decreased %NS of IWG, indicating that white clover would rely more on N in fertilizers than on symbiotic N_2 fixation if an excessive amount of N fertilizer was applied, which could impair the complementary effect in IWG and WC intercrops, resulting in inefficient utilization of N resources. White clover as a service crop could supply sufficient N for IWG intercrops under appropriate soil N fertility and species-relative frequencies.

Author Contributions: Conceptualization, S.L., E.S.J., L.-M.D.M., and Y.Z.; methodology, S.L. and E.S.J.; software, S.L.; formal analysis, S.L.; investigation, S.L.; resources, L.-M.D.M. and N.L.; data curation, S.L.; writing—original draft preparation, S.L.; writing—review and editing, E.S.J., L.-M.D.M., Y.Z., and N.L.; supervision, Y.Z. and L.-M.D.M.; project administration, L.-M.D.M.; funding acquisition, Y.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the China Forage and Grass Research System (CARS-34).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors gratefully acknowledge the Land Institute for supplying the seeds of intermediate wheatgrass used for experiments. We would like to thank Jan-Eric Englund for help with data analysis. We are grateful to Yuqi Wei, Tao Li, and Ye Wu (China Agricultural University) for helping us collect plant and soil samples. We also thank the anonymous reviewers for their careful reading of our manuscript and their many insightful comments and suggestions.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Smaje, C. The strong perennial vision: A critical review. *Agroecol. Sust. Food* **2015**, *39*, 471–499. [CrossRef]
2. Pimentel, D.; Cerasale, D.; Stanley, R.C.; Perlman, R.; Newman, E.M.; Brent, L.C.; Mullan, A.; Chang, D.T.-I. Annual vs. perennial grain production. *Agric. Ecosyst. Environ.* **2012**, *161*, 1–9. [CrossRef]
3. Culman, S.W.; Snapp, S.S.; Ollenburger, M.; Basso, B.; DeHaan, L.R. Soil and water quality rapidly responds to the perennial grain Kernza wheatgrass. *Agron. J.* **2013**, *105*, 735–744. [CrossRef]
4. Wayman, S.; Debray, V.; Parry, S.; David, C.; Ryan, M.R. Perspectives on perennial grain crop production among organic and conventional farmers in France and the United States. *Agriculture* **2019**, *9*, 244. [CrossRef]
5. Jungers, J.M.; DeHaan, L.H.; Mulla, D.J.; Sheaffer, C.C.; Wyse, D.L. Reduced nitrate leaching in a perennial grain crop compared to maize in the Upper Midwest, USA. *Agric. Ecosyst. Environ.* **2019**, *272*, 63–73. [CrossRef]
6. Dick, C.; Cattani, D.; Entz, M.H. Kernza intermediate wheatgrass (*Thinopyrum intermedium*) grain production as influenced by legume intercropping and residue management. *Can. J. Plant Sci.* **2018**, *98*, 1376–1379. [CrossRef]
7. Lithourgidis, A.S.; Dordas, C.A.; Vlachostergios, D.N. Annual intercrops an alternative pathway for sustainable agriculture. *Aust. J. Crop Sci.* **2011**, *5*, 396–410.
8. Jensen, E.S.; Carlsson, G.; Hauggaard-Nielsen, H. Intercropping of grain legumes and cereals improves the use of soil N resources and reduces the requirement for synthetic fertilizer N: A global-scale analysis. *Agron. Sustain. Dev.* **2020**, *40*, 1–9. [CrossRef]
9. Hartwig, N.L.; Ammon, H.U. Cover crops and living mulches. *Weed Sci.* **2002**, *50*, 688–699. [CrossRef]

10. Ogilvie, C.M.; Deen, W.; Martin, R.C. Service crop management to maximize crop water supply and improve agroecosystem resilience: A review. *J. Soil Water Conserv.* **2019**, *74*, 389–404. [CrossRef]
11. Hayes, R.C.; Newell, M.T.; Crews, T.E.; Peoples, M.B. Perennial cereal crops: An initial evaluation of wheat derivatives grown in mixtures with a regenerating annual legume. *Renew. Agr. Food Syst.* **2016**, *32*, 276–290. [CrossRef]
12. Tautges, N.E.; Jungers, J.M.; DeHaan, L.R.; Wyse, D.L.; Sheaffer, C.C. Maintaining grain yields of the perennial cereal intermediate wheatgrass in monoculture v. bi-culture with alfalfa in the Upper Midwestern USA. *J. Agric. Sci.* **2018**, *156*, 758–773. [CrossRef]
13. Favre, J.R.; Castiblanco, T.M.; Combs, D.K.; Wattiaux, M.A.; Picasso, V.D. Forage nutritive value and predicted fiber digestibility of Kernza intermediate wheatgrass in monoculture and in mixture with red clover during the first production year. *Anim. Feed Sci. Technol.* **2019**, *258*, 114298. [CrossRef]
14. Li, S.; Barreiro, A.; Jensen, E.S.; Zhang, Y.; Mårtensson, L.-M.D. Early interspecific dynamics, dry matter production and nitrogen use in Kernza (Thinopyrum intermedium)—alfalfa (*Medicago sativa* L.) mixed intercropping. *Acta Agric. Scand. Sect. B Soil Plant Sci.* **2019**, *70*, 165–175. [CrossRef]
15. Dick, C. An investigation into ecological farming systems on the Canadian prairies. Master's Thesis, University of Manitoba, Winnipeg, MB, Canada, 2016.
16. Wagoner, P.; Schaeffer, J.R. Perennial grain development: Past efforts and potential for the future. *Crit. Rev. Plant Sci.* **1990**, *9*, 381–408. [CrossRef]
17. Carlsson, G.; Huss-Danell, K. Nitrogen fixation in perennial forage legumes in the field. *Plant Soil* **2003**, *253*, 353–372. [CrossRef]
18. Frankow-Lindberg, B.E.; Dahlin, A.S. N_2 fixation, N transfer, and yield in grassland communities including a deep-rooted legume or non-legume species. *Plant Soil* **2013**, *370*, 567–581. [CrossRef]
19. Louarn, G.; Pereira-Lopès, E.; Fustec, J.; Mary, B.; Voisin, A.-S.; de Faccio Carvalho, P.C.; Gastal, F. 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* **2014**, *389*, 289–305. [CrossRef]
20. Pirhofer-Walzl, K.; Rasmussen, J.; Høgh-Jensen, H.; Eriksen, J.; Søegaard, K.; Rasmussen, J. Nitrogen transfer from forage legumes to nine neighbouring plants in a multi-species grassland. *Plant Soil* **2011**, *350*, 71–84. [CrossRef]
21. Thilakarathna, M.S.; McElroy, M.S.; Chapagain, T.; Papadopoulos, Y.A.; Raizada, M.N. Belowground nitrogen transfer from legumes to non-legumes under managed herbaceous cropping systems. A review. *Agron. Sustain. Dev.* **2016**, *36*, 1–16. [CrossRef]
22. Unkovich, M.; Herridge, D.; Peoples, M.; Cadisch, G.; Boddey, B.; Giller, K.; Alves, B.; Chalk, P. *Measuring Plant-Associated Nitrogen Fixation in Agricultural Systems*; Australian Centre for International Agricultural Research (ACIAR): Canberra, Australia, 2008; p. 258.
23. Hauggaard-Nielsen, H.; Andersen, M.K.; Jørnsgaard, B.; Jensen, E.S. Density and relative frequency effects on competitive interactions and resource use in pea–barley intercrops. *Field Crop. Res.* **2006**, *95*, 256–267. [CrossRef]
24. Olorunmaiye, P.M.; Lagoke, S.T.; Adigun, J.A.; Orija, O.R. Effect of intercropping with maize on weed diversity in cassava. *Environ. Exp. Biol.* **2013**, *11*, 189–193.
25. Lithourgidis, A.S.; Vasilakoglou, I.B.; Dhima, K.V.; Dordas, C.A.; Yiakoulaki, M.D. Forage yield and quality of common vetch mixtures with oat and triticale in two seeding ratios. *Field Crop. Res.* **2006**, *99*, 106–113. [CrossRef]
26. Arlauskiene, A.; Maiksteniene, S.; Sarunaite, L.; Kadziuliene, Z.; Deveikyte, I.; Zekaite, V.; Cesnuleviciene, R. Competitiveness and productivity of organically grown pea and spring cereal intercrops. *Zemdirbyste* **2011**, *98*, 339–348.
27. Jensen, E.S. Grain yield, symbiotic N_2 fixation and interspecific competition for inorganic N in pea-barley intercrops. *Plant Soil* **1996**, *182*, 25–38. [CrossRef]
28. Fan, Z.; Zhao, Y.; Chai, Q.; Zhao, C.; Yu, A.; Coulter, J.A.; Gan, Y.; Cao, W. Synchrony of nitrogen supply and crop demand are driven via high maize density in maize/pea strip intercropping. *Sci. Rep.* **2019**, *9*, 10954. [CrossRef]
29. Neumann, A.; Werner, J.; Rauber, R. Evaluation of yield–density relationships and optimization of intercrop compositions of field-grown pea–oat intercrops using the replacement series and the response surface design. *Field Crop. Res.* **2009**, *114*, 286–294. [CrossRef]
30. Chalk, P.M.; Peoples, M.B.; McNeill, A.M.; Boddey, R.M.; Unkovich, M.J.; Gardener, M.J.; Silva, C.F.; Chen, D. Methodologies for estimating nitrogen transfer between legumes and companion species in agro-ecosystems: A review of ^{15}N-enriched techniques. *Soil Biol. Biochem.* **2014**, *73*, 10–21. [CrossRef]
31. Frahm, C.S.; Tautges, N.E.; Jungers, J.M.; Ehlke, N.J.; Wyse, D.L.; Sheaffer, C.C. Responses of intermediate wheatgrass to plant growth regulators and nitrogen fertilizer. *Agron. J.* **2018**, *110*, 1028–1035. [CrossRef]
32. Jungers, J.M.; DeHaan, L.R.; Betts, K.J.; Sheaffer, C.C.; Wyse, D.L. Intermediate wheatgrass grain and forage yield responses to nitrogen fertilization. *Agron. J.* **2017**, *109*, 462–472. [CrossRef]
33. Fernandez, C.W.; Ehlke, N.; Sheaffer, C.C.; Jungers, J.M. Effects of nitrogen fertilization and planting density on intermediate wheatgrass yield. *Agron. J.* **2020**, *112*, 4159–4170. [CrossRef]
34. Ghaley, B.B.; Hauggaard-Nielsen, H.; Høgh-Jensen, H.; Jensen, E.S. Intercropping of wheat and pea as influenced by nitrogen fertilization. *Nutr. Cycl. Agroecosyst.* **2005**, *73*, 201–212. [CrossRef]
35. Li, Y.; Liu, H.; Huang, G.; Zhang, R.; Yang, H. Nitrate nitrogen accumulation and leaching pattern at a winter wheat: Summer maize cropping field in the North China Plain. *Environ. Earth Sci.* **2016**, *75*, 118. [CrossRef]
36. Jørgensen, F.V.; Jensen, E.S.; Schjoerring, J.K. Dinitrogen fixation in white clover grown in pure stand and mixture with ryegrass estimated by the immobilized ^{15}N isotope dilution method. *Plant Soil* **1999**, *208*, 293–305. [CrossRef]

37. Thilakarathna, M.S.; Papadopoulos, Y.A.; Rodd, A.V.; Grimmett, M.; Fillmore, S.A.E.; Crouse, M.; Prithiviraj, B. Nitrogen fixation and transfer of red clover genotypes under legume–grass forage based production systems. *Nutr. Cycl. Agroecosyst.* **2016**, *106*, 233–247. [CrossRef]
38. FAO/IAEA Agriculture and Biotechnology Laboratory and Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture. *Use of Isotope and Radiation methods in Soil and Water Management and Crop Nutrition*; International Atomic Energy Agency: Vienna, Austria, 2001; pp. 27–36.
39. Izaurralde, R.C.; McGill, W.B.; Juma, N.G. Nitrogen fixation efficiency, interspecies N transfer, and root growth in barley-field pea intercrop on a Black Chernozemic soil. *Biol. Fert. Soils* **1992**, *13*, 11–16. [CrossRef]
40. Somado, E.A.; Kuehne, R.F. Appraisal of the ^{15}N-isotope dilution and ^{15}N natural abundance methods for quantifying nitrogen fixation by flood-tolerant green manure legumes. *Afr. J. Biotechnol.* **2006**, *5*, 1210–1214.
41. Willey, R.W.; Rao, M.R. A competitive ratio for quantifying competition between intercrops. *Exp. Agric.* **2008**, *16*, 117–125. [CrossRef]
42. Hunter, M.C.; Sheaffer, C.C.; Culman, S.W.; Jungers, J.M. Effects of defoliation and row spacing on intermediate wheatgrass I: Grain production. *Agron. J.* **2020**, *112*, 1748–1763. [CrossRef]
43. Bedoussac, L.; Journet, E.-P.; Hauggaard-Nielsen, H.; Naudin, C.; Corre-Hellou, G.; Jensen, E.S.; Prieur, L.; Justes, E. Ecological principles underlying the increase of productivity achieved by cereal-grain legume intercrops in organic farming. A review. *Agron. Sustain. Dev.* **2015**, *35*, 911–935. [CrossRef]
44. Rao, M.R.; Rego, T.J.; Willey, R.W. Response of cereals to nitrogen in sole cropping and intercropping with different legumes. *Plant Soil* **1987**, *101*, 167–177. [CrossRef]
45. Ledgard, S.F.; Steele, K.W. Biological nitrogen fixation in mixed legume/grass pastures. *Plant Soil* **1992**, *141*, 137–153. [CrossRef]
46. Høgh-Jensen, H.; Schjorring, J.K. Interactions between white clover and ryegrass under contrasting nitrogen availability: N_2 fixation, N fertilizer recovery, N transfer and water use efficiency. *Plant Soil* **1997**, *197*, 187–199. [CrossRef]
47. Gylfadóttir, T.; Helgadóttir, Á.; Høgh-Jensen, H. Consequences of including adapted white clover in northern European grassland: Transfer and deposition of nitrogen. *Plant Soil* **2007**, *297*, 93–104. [CrossRef]
48. Fustec, J.; Lesuffleur, F.; Mahieu, S.; Cliquet, J.-B. Nitrogen rhizodeposition of legumes. A review. *Agron. Sustain. Dev.* **2010**, *30*, 57–66. [CrossRef]
49. Genard, T.; Etienne, P.; Laine, P.; Yvin, J.C.; Diquelou, S. Nitrogen transfer from *Lupinus albus* L., *Trifolium incarnatum* L. and *Vicia sativa* L. contribute differently to rapeseed (*Brassica napus* L.) nitrogen nutrition. *Heliyon* **2016**, *2*, e00150. [CrossRef] [PubMed]
50. Boller, B.C.; Nösberger, J. Symbiotically fixed nitrogen from field-grown white and red clover mixed with ryegrasses at low levels of ^{15}N-fertilization. *Plant Soil* **1987**, *104*, 219–226. [CrossRef]
51. Kendall, W.A.; Stringer, W.C. Physiological aspects of clover. In *Clover Science and Technology*; Taylor, N.L., Ed.; American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America: Madison, WI, USA, 1985; Volume 25, pp. 111–159. [CrossRef]

Agronomy for Sustainable Development (2022) 42: 21
https://doi.org/10.1007/s13593-022-00752-0

RESEARCH ARTICLE

Agronomic performance, nitrogen acquisition and water-use efficiency of the perennial grain crop *Thinopyrum intermedium* in a monoculture and intercropped with alfalfa in Scandinavia

Linda-Maria Dimitrova Mårtensson[1] · **Ana Barreiro**[1] · **Shoujiao Li**[1] · **Erik Steen Jensen**[1]

Accepted: 5 January 2022

Abstract
The perennial forage grass *Thinopyrum intermedium* (Host) Barkworth & Dewey, commonly known as intermediate wheatgrass (IWG) or by the commercial name Kernza™, is being developed as a perennial grain crop, i.e. being bred for its improved agronomic performance and food qualities. Intercropping legumes and grasses is a strategy for improving resource use and sustainability in cropping systems. Here, we show for the first time the agronomic performance of IWG as a perennial cereal grown as a monocrop and as an intercrop (alternate row, 0.5:0.5) with *Medicago sativa* L. (alfalfa/lucerne) in southern Sweden. The seeds of cycle 3 IWG were accessed from The Land Institute (TLI) of Salinas, Kansas, USA, and used to establish a local seed production plot (in 2014) for the establishment of the perennial systems (in 2016) utilised in this study. Both the monocrop and intercrop were sown with 25 cm row spacing with alternate rows of IWG and alfalfa in the intercrop (i.e. replacement design) with unknown sowing density. Intercropping provided sustained IWG grain production under the dry conditions of 2018, but also in the following year. This was evidently associated with a higher nitrogen accumulation in intercropped practice. Thus, intercropping seems to have stabilised the IWG grain production in the dry conditions of 2018, when the grain production in the intercrop was similar to that of the monocrop in the same year. This result was further supported by the lower discrimination against ^{13}C (as an indicator of water use efficiency) in the intercrop components compared to the sole crop in 2018. The lower discrimination indicates high water use efficiency in the intercropped IWG in comparison to the IWG in monoculture, and we conclude that intercropping perennial cereal grain crops with legumes provides better growing conditions in terms of nitrogen acquisition, and water status, to cope with more extreme drought spells expected from climate change.

Keywords Intermediate wheatgrass · Grain yield · Straw yield · Drought · N content · N_2 fixation · Lucerne

1 Introduction

Perennial crops represent a paradigm shift in agriculture and have the potential to contribute to increased sustainability of production systems (Crews et al. 2018; FAO 2013). Perennial cereal grain crops are more robust and multifunctional than annual crops (Ryan et al. 2018). In the pursuit for suitable candidates for the development of perennial grain cereal crops, the perennial forage grass *Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey, commonly known as intermediate wheatgrass (henceforward referred to as IWG), was selected for domestication in 1983 (Wagoner and Schauer 1990) and included in a breeding programme for perennial cereal grain production in 2002 (DeHaan et al. 2013) and trademarked under the name Kernza™ (Fig. 1). The selection was based on flavour, ease of threshing, large seed size, resistance to shattering, lodging resistance, ease of harvest and perennial growth, and was identified as the most promising species among 100 other perennial grasses (Wagoner and Schauer 1990). While plant breeding improves grain yields, it has been suggested also to focus on the crop multifunctionality, which perennial cereal may provide besides grain production (Duchene et al. 2019). Potential multiple functions from perennial cereals include feed and forage production, protection and regeneration of soil quality, reduced nutrient losses, reduced requirements for agrochemicals, climate change adaptation and mitigation, conservation of biodiversity and

---

✉ Linda-Maria Dimitrova Mårtensson
Linda.Maria.Martensson@slu.se

[1] Department of Biosystems and Technology, Swedish University of Agricultural Sciences, P.O Box 103, 230 53 Alnarp, Sweden

Fig. 1 Intermediate wheatgrass Kernza™ grains after harvest and threshing in Sweden, 2017. Photograph courtesy of Ryan Davidson.

improved agroecosystem resilience (Ryan et al. 2018). Taking the landscape perspective into account, multifunctionality is vital and the inclusion of elements of perennial crops in the production landscape may provide a higher delivery of several ecosystem services beyond the provisioning service of crop yields (Asbjornsen et al. 2014; Landis 2017) (Fig. 2).

The inclusion of legumes in the cropping system provides a wider range of ecosystem services like the increase in the N use efficiency (NUE) (Jensen et al. 2020), leading to a more sustainable agricultural production. Moreover, biological N_2 fixation reduces the need for synthetic N fertilisers (Jensen et al. 2020) and reduces environmental costs related to fertiliser production, transportation and use, not the least in terms of climate change (Jensen et al. 2012). Intercropping IWG with perennial legumes makes atmospheric N_2 available to the production system via symbiotic N_2 fixation. With time, fixed N in legume residues and exudates are mineralised and made available to the perennial cereal (Crews et al. 2016), while N also may be transferred from legumes via mycorrhizal networks (Johansen and Jensen 1996; Thilakarathna et al. 2016). Niche complementarity is a well-known mechanism driving coexistence resulting in potential over-yielding (Gross et al. 2007). This mechanism often erases or supplements the mutual competition pressure between the legume and cereal crops. It is well-known that intercropping cereals and grain legumes result in higher and more stable grain yields, and a higher cereal protein concentration compared to the sole crop cereals (Bedoussac et al. 2015). However, for the novel production systems including IWG, studies on intercropping in Kernza production are sparse and results contradictory. While Dick et al. (2018) did not find and effect of intercropping with alfalfa (*Medicago sativa*), sweet clover (*Melilotus officinalis*) and white clover (*Trifolium repens*) on Kernza grain production, Tautges et al. (2018) found that the yield loss with stand age was reduced when IWG was intercropped with alfalfa (*Medicago sativa*). Furthermore, IWG has been found to provide suitable forage for beef and dairy cows, as well as for growing heifers, and that intercropping IWG with red clover (*Trifolium pratense*) increased the forage nutritive value in the fall (Favre et al. 2019).

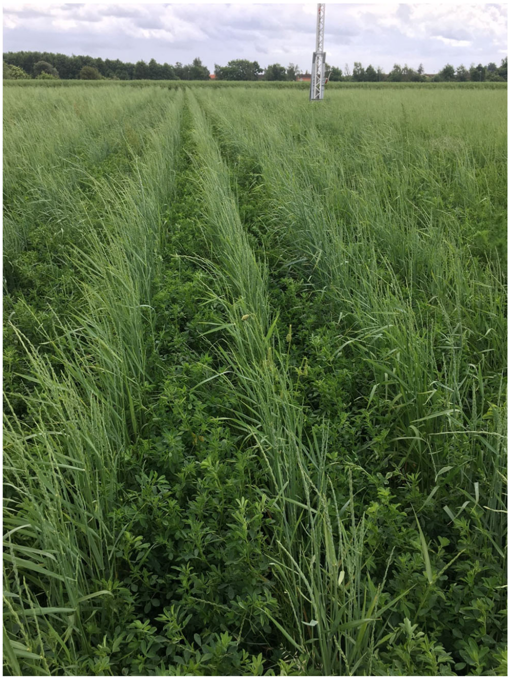


Fig. 2 Intermediate wheatgrass (*Thinopyrum intermedium*) and alfalfa (*Medicago sativa* L. cv. Power 4.2) in 2016 at the SITES Lönnstorp Research Station, Swedish University of Agricultural Sciences (SLU), Alnarp, Sweden. Photograph by Erik Steen Jensen.

Intermediate wheatgrass has been shown to tolerate partial-season irrigation deficits better than other perennial grasses (Orloff et al. 2016) and to maintain a relatively high water use efficiency during the growing season (Culman et al. 2013; Oliveira et al. 2019). This is an important feature in the current climatic conditions, where drought events are expected to increase in frequency and severity in southern Europe (Roudier et al. 2016), but also for northern Europe (SMHI 2019). The performance of perennial grasses can be related to the capability of sustained aboveground biomass production under dry conditions through enhanced water use efficiency (WUE), which has been proven to occur in C_3 grasses (Kørup et al. 2018), often resulting from the response mechanism of reducing discrimination against ^{13}C in photosynthesis (Mårtensson et al. 2017). In addition, the larger root system of IWG allows access to water in deeper soil layers, while the annual crops do not have access to these resources (Vico and Brunsell 2018). Dry growing conditions make the soil nutrients, especially N, immobile, resulting in reduced

NUE. Indeed, the relationship between crop water use, WUE and grain NUE has been confirmed (Dalal et al. 2013).

To our knowledge, research on agronomy and nitrogen nutrition in IWG and Kernza grain production has not yet been carried out in Scandinavia to any large extent, neither in sole cropping nor in intercropping with perennial legumes. This study is the first one to determine the agronomic performance and nitrogen acquisition of IWG when grown under the temperate climate of southern Sweden with and without a perennial legume companion/service crop during the initial three years. The study also demonstrate the capability of sustained aboveground biomass production under dry conditions through enhanced water use efficiency as indicated by the downregulation of ^{13}C discrimination. To elucidate the effect of intercropping on IWG, we pose the following hypothesis:

> Intercropping IWG with alfalfa will increase IWG grain and straw biomass yields, N concentrations and N accumulated in the IWG biomass, as well as reduce the discrimination against ^{13}C in IWG under dry conditions.

2 Methods and materials

2.1 Experimental design

The SITES (Swedish Infrastructure for Ecosystem Science) Agroecological Field Experiment (SAFE), is a long-term south Swedish cropping system large-scale experimental facility, was established in 2016 on previously conventionally managed land. The SAFE is located at the SITES Lönnstorp Research Station, SLU, in Alnarp (55.65° N, 13.06° E) in a region with a humid continental climate (Fig. 3). The soil type is a sandy loam soil (67% sand, 18% clay), with a soil pH$_{H2O}$ (0–30 cm) of 7.3, and 0.9% soil organic matter content. Soil nutrient conditions for the site correspond to 51 mg kg^{-1} of aluminium lactate extractable P, 0.36 g kg^{-1} total P, 65 mg kg^{-1} aluminium lactate extractable K, 1.4 g kg^{-1} total K and a total N content of 0.2 %. The SAFE has a block design with spatially contained blocks where the geographical layout was guided by initial measurements on soil variables (pH, moisture, nitrogen levels, etc.) to ensure similar conditions within the blocks. The SAFE includes a perennial cereal grain system, representing a model for future potential perennial cereal grain production under low input organic management. The perennial cereal grain system in SAFE holds a monocrop with IWG and an intercrop with IWG and alfalfa (*Medicago sativa* cv. Power 4.2; Fig. 2) in large plots (48 × 50 m). Seeds of intermediate wheatgrass were accessed from the cycle 3 (2014) germplasm of the perennial grain breeding program of The Land Institute (TLI) of Salinas, Kansas, USA (Zhang et al. 2016). The seeds were used to establish a local seed production plot (5 kg sown on 3000 m^2 in September 2014) for the establishment of the perennial systems in SAFE.

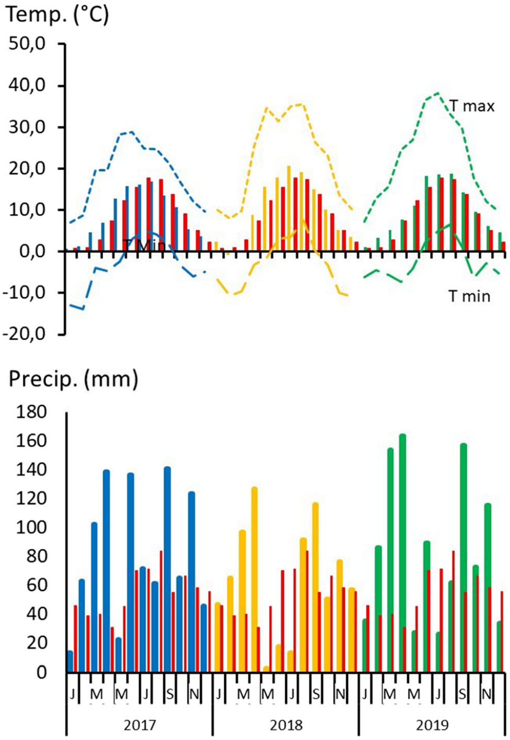


Fig. 3. The monthly mean (bars), monthly minimum (dashed lines), monthly maximum (dotted lines) and 30-year mean temperature (thin bars) temperatures (°C). The monthly (bars) and the 30-year (thin bars) mean precipitations (mm). Colour codes for bars and lines: blue for 2017, yellow for 2018, green for 2019, and for red the 3-year means. Site-specific climate conditions at the SAFE facility are collected by the *in situ* automatic weather station and retrieved from the SITES data portal (https://data.fieldsites.se/portal/). Abbreviations: Temp., temperature; T MEAN, monthly mean temperature; T MIN, monthly minimum temperature; T MAX, monthly maximum temperature; Precip. precipitation.

The IWG sole crop and IWG-alfalfa intercrop was sown in May 2016 with complementary sowing in September 2016 (Table 1). Both the sole crop and the intercrop were sown with 25 cm row spacing with alternate rows of IWG and alfalfa in the intercrop (i.e. replacement design). The IWG sowing density was not possible to record, due to faults in the sowing equipment, which also led to the complementary sowing September 2016. The density was estimated to be approximately 17 kg ha^{-1}. The sowing density of alfalfa inoculated with *Sinorhizobium meliloti* was 8 kg ha^{-1}. In 2017, the IWG and IWG-alfalfa intercrop was fertilised using 444 kg Biofer® ha^{-1} year^{-1} (Gyllebo gödning AB, Malmö, Sweden; Biofer is

Table 1 Management activities in the model system for perennial cereal production. Abbreviation *IWG*, intermediate wheatgrass. The asterisk (*) indicates a missing date in the experimental log, and † indicates interrupted work due to heavy rain.

	Month(s), year(s)
Harrowing (approx. 5 cm depth)	5 Apr 2016
Harrowing (approx. 4 cm depth)	19 Apr 2016
Sowing IWG and alfalfa	2–3 May 2016
Complementary sowing IWG	24 May 2016
Topping	25 Jul 2016
Complementary sowing IWG	Sep 2016*
Topping	25 Nov 2016
Fertilisation (Biofer, see text)	4 May 2017
Row cultivation	5 May 2017
Cutting alfalfa (with trimmer)	5 May 2017
Hand harvest, i.e. sampling for analysis	19–20 Sep 2017
Full harvest	13 Nov 2017
Row cultivation (block C)†	26 Apr 2018
Row cultivation (blocks A, B, D)	14–15 May 2018
Fertilisation (digestate, see text)	18 May 2018
Hand harvest, i.e. sampling for analysis	16 Aug 2018
Full harvest	3 Sep 2018
Fertilisation (digestate, see text)	9–10 May 2019
Hand harvest, i.e. sampling for analysis	Sep 2019*
Full harvest	16 Sep 2019

certified for organic farming) (444 kg Biofer correspond to 40 kg N, 12 kg P and 4 kg K ha^{-1} year^{-1}). In 2017, row crop cultivator was used for mechanical weeding in the IWG sole crop. The alfalfa in the IWG-alfalfa intercrop was cut in May 2017 to restrict alfalfa from overgrowing the IWG and the alfalfa residues were left to decompose in the rows as a green manure. In 2018 and 2019, 17 tonnes of biogas digestate per hectare^{-1}, corresponding to 35-40 kg N ha^{-1}, were applied as fertiliser to the IWG sole crop and intercrop. No weed management substance was applied in 2018 and 2019.

2.2 Sampling and analyses

Aboveground plant material was sampled in four 0.25 m^2 subplots in each experimental plot before harvest in 2017, 2018 and 2019. The grain and biomass yield of IWG, the biomass yield of alfalfa and the biomass of weeds were determined after threshing and drying (65 °C, 48 h). The dry matter harvest index of IWG was calculated as the percentage grain yield of total dry matter, and the nitrogen harvest index was calculated as the percentage grain N accumulation of total N accumulation. The proportion of C and N, and the isotopic composition of ^{13}C and ^{15}N, was analysed on dried (65 °C, 48 h), milled (<1 mm) plant material using Dumas combustion on an elemental analyser (CE 1110, Thermo Electron, Milan, Italy) coupled in continuous flow mode to a Finnigan MAT Delta PLUS isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany). The isotopic analysis was done at the Department of Geosciences and Natural Resource Management at the University of Copenhagen.

2.3 Land equivalent ratio

The Land Equivalent Ratio, LER (Willey and Osiru 1972), is the area of legume and cereal monocrops required to produce the same amount of grain as one unit area of the cereal–legume intercrop. Since no monocrop of alfalfa was available, the partial Land Equivalent Ratio of IWG, $p\mathrm{LER}_{\mathrm{IWG}}$, was calculated as the ratio between IWG grain dry matter yield in the monocrop and the intercrop (Eq. 1).

$$pLER_{IWG} = \frac{Y_{IWG\ grain\ of\ monocrop}}{Y_{IWG\ grain\ of\ intercrop}} \tag{1}$$

2.4 Nitrogen fixation and soil N acquisition in alfalfa

The ^{15}N abundance in legume and non-legume samples, expressed as δ^{15}N (‰ deviation from the ^{15}N abundance in atmospheric N_2; Unkovich et al. 2008), was used to calculate the proportion (%Ndfa; Eq. 2) and the amount (N_{FIX}; Eq. 3) of N in the aboveground legume biomass that was derived from biological N_2 fixation.

$$\%Ndfa = \frac{\delta^{15}N_{reference} - \delta^{15}N_{legume}}{\delta^{15}N_{reference} - B} \times 100 \tag{2}$$

$$N_{FIX}\left(\mathrm{kg\ N\ ha^{-1}}\right) = N_{YIELD}\left(\mathrm{kg\ N\ ha^{-1}}\right) \times \frac{\%Ndfa}{100} \tag{3}$$

The mean δ^{15}N value of the IWG and weed samples from each experimental plot was used as $\delta^{15}\mathrm{N}_{\mathrm{reference}}$ to calculate %Ndfa in the legumes present in the same plot. The B value for alfalfa, i.e. the δ^{15}N in the legume when relying on N_2 fixation as its only N source, was −0.677 (Unkovich et al. 2008), and is included in the equation to account for discrimination against ^{15}N during N_2 fixation and N translocation within the legume plant (Högberg 1997). The values of legume N accumulation (N concentration multiplied by biomass dry weight) and %Ndfa were used to calculate the amount of legume N derived from N_2 fixation (Eq. 3). The amount of N derived from the soil and fertiliser (N_{SOIL}) constitutes the remaining proportion N acquired (Eq. 4).

$$N_{SOIL}\left(\mathrm{kg\ N\ ha^{-1}}\right) = N_{YIELD}\left(\mathrm{kg\ N\ ha^{-1}}\right) \times (1-\%Ndfa) \tag{4}$$

2.5 Discrimination against ^{13}C

Natural abundance of ^{13}C was used to calculate the discrimination against ^{13}C (Δ^{13}C), which is positively related with water use efficiency (Farquhar et al. 1982; Farquhar et al. 1989; Farquhar and Richards 1984; O'Leary 1981). The ^{13}C composition (δ^{13}C) is given by the measured ratio of ^{13}C/^{12}C and the Vienna Pee Dee Belemnite reference material, where R_{PDB} = 0.01117960 (Coplen 2011) (Eq. 5) (Farquhar et al. 1982; O'Leary 1981; O'Leary 1988; Park and Epstein 1960). The discrimination against ^{13}C was calculated (Eq. 6), using δ_{atmos} and δ_{plant} which refer to the carbon isotope composition of the atmospheric CO_2 and plant material, respectively. The carbon isotope composition of the atmospheric CO_2, δ_{atmos}, is approximately −8.0‰ (Farquhar et al. 1989; O'Leary 1988).

$$\delta^{13}C_{PDB}\ (‰) = \left(\frac{R_{sample}}{R_{PDB}} - 1\right) \times 1000 \qquad (5)$$

$$\Delta^{13}C\ (‰) = \frac{(\delta_{atmos} - \delta_{plant})}{1 + \delta_{atmos}/1000} \qquad (6)$$

2.6 Statistics

The effects of intercropping on the grain and straw yields, the N concentrations in dry matter, the N accumulated in the aboveground biomass and the discrimination against 13C were statistically analysed using a factorial design with repeated measures. In the model, block was a random factor and the 'crop', 'year' and the interaction between 'crop' and 'year' were fixed factors with the correlation structure AR(1) for observations from the same block and 'crop'. The analysis was done using proc MIXED in SAS (SAS 9.4) with Kenward-Rogers method for denominator degrees of freedom. Tukey's post hoc test at the $p < 0.05$ level of significance was used for pairwise differences between treatment levels. The interaction 'crop*year' was significant in all analyses except for intermediate wheatgrass straw yield, harvest index and nitrogen concentration in intermediate wheatgrass grain. The amount of N_2 fixation in legumes was analysed with only 'year' as fixed factor. Bivariate parametric Pearson correlation tests, with $p < 0.05$ as the level of significance, were run to explore the relationships between discrimination against ^{13}C, yield, N concentrations and N accumulation. The correlations were run on data separated by year (2017, 2018 and 2019), cropping system (monocrop and intercrop) and yield fractions (grain and straw). The correlations were performed with IBM Statistics SPSS software.

3 Results and discussion

3.1 Dry matter production

The IWG grain dry matter yield varied between 0.26 and 0.88 t ha^{-1} in the monocrop and between 0.28 and 0.55 t ha^{-1} in the intercrop (Fig. 4). The IWG grain dry matter yield was significantly higher in the monocrop than the intercrop in 2017, and significantly greater in 2017 than during the subsequent 2 years in both the monocrop and the intercrop (Fig. 4). The IWG straw biomass did not follow the same pattern as the IWG grain, but was rather stable throughout the 3 years. The IWG straw dry matter yield varied between 5.4 and 8.2 t ha^{-1} in the sole crop and between 4.6 and 5.3 ha^{-1} in the intercrop (Fig. 4). In the IWG monocrop, the IWG straw dry matter yield was significantly lower in the dry year 2018 and then in 2017 and 2019, while in the intercrop, IWG straw biomass was similar over all three experimental years. The alfalfa biomass yield varied between 3.8 and 4.5 t ha^{-1} and did not differ significantly between the experimental years. The IWG grain partial land equivalent ratio in the intercrop was higher in 2018 and 2019 than in 2017 (Table 2). The harvest index (HI) of IWG was highest in both crops in 2017, with declining values over the 3 experimental years (Table 2).

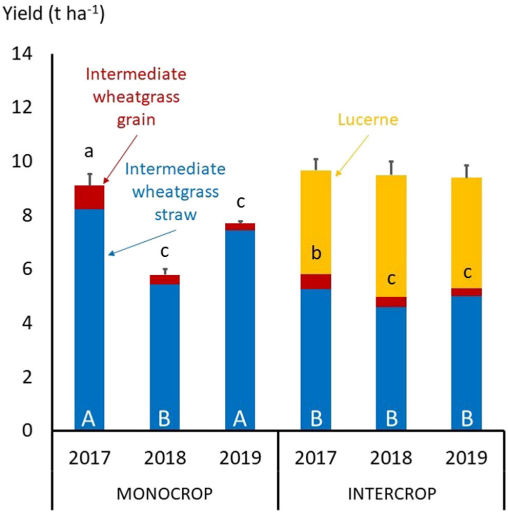


Fig. 4 The aboveground dry matter biomass production of intermediate wheatgrass grain (red bars), intermediate wheatgrass straw (blue bars) and lucerne (yellow bars). Different black lower case letters (above red bars) indicate differences in intermediate wheatgrass grain yield, white upper case letters (within blue bars) indicate differences in intermediate wheatgrass straw yield, from proc MIXED in SAS (SAS 9.4) with Kenward-Rogers method for denominator degrees of freedom, using univariate ANOVA with Tukey's post hoc test at the $p < 0.05$ level of significance.

Table 2 The partial Land Equivalent Ratio for IWG ($p\mathrm{LER}_{\mathrm{IWG}}$), harvest index (HI, %) and nitrogen harvest index (NHI, %) of IWG presented as means ($N = 4$) with standard errors. Different lowercase letters indicate differences between treatments (crop and year) from proc MIXED in SAS (SAS 9.4) with Kenward-Rogers method for denominator degrees of freedom and Tukey's post hoc test at the $p < 0.05$ level of significance (SAS 9.4). Abbreviation IWG, intermediate wheatgrass.

	$pLER_{IWG}$	*HI* [%]	*NHI* [%]
IWG monocrop			
2017	n. a.	9.60 ± 0.39***a***	23.3 ± 2.4***ab***
2018	n. a.	6.23 ± 0.87***bc***	25.2 ± 2.8***a***
2019	n. a.	3.38 ± 0.20***d***	16.5 ± 2.3***bc***
IWG intercrop			
2017	0.63 ± 0.049***b***	9.55 ± 1.3***a***	14.8 ± 3.0***c***
2018	1.08 ± 0.16***a***	7.80 ± 0.89***ab***	26.3 ± 2.3***a***
2019	1.07 ± 0.11***a***	5.38 ± 1.0***c***	19.8 ± 4.2***abc***

The levels and pattern of the IWG grain yields are in coherence with what other reports (Hunter et al. 2020; Jungers et al. 2019; Tautges et al. 2018), despite the comparably low amount of fertiliser used in our low-input management design. The reported IWG grain dry matter production levels are mainly stemming from the IWG germplasm coming from the TLI third cycle of IWG, which is quite an early line in the IWG breeding programme. The programme is constantly making progress towards higher seed yield (DeHaan et al. 2013) together with other important agronomic features (Wagoner and Schauer 1990). In parallel to a higher grain yield, the longevity of IWG grain production is also questioned. The decline in grain yield over years may be reduced under high fertilisation rates (120–160 kg N ha^{-1}) (Culman et al. 2013; Jungers et al. 2019), but may be simultaneously associated with stronger environmental impacts through leaching and N_2O emissions. However, the large and deep root system of IWG (Sprunger et al. 2019) and the high capability of IWG to exploit the soil volume (Duchene et al. 2020), compared to annual crops, may reduce the leaching risk to a minimum (Culman et al. 2013; Jungers et al. 2019).

The higher IWG grain partial land equivalent ratio in the intercrop in 2018 and 2019, than in 2017, indicates the better growing conditions for IWG when intercropped with alfalfa. The increasing amount of N becoming available from the alfalfa over time, together with a lower density, and thus lower intraspecific competition, of IWG in the intercrop may also positively influence the performance of IWG in the intercrop compared to in the monocrop. The HI follows the pattern of declining grain yield over years, which is in line with the findings from Hunter et al. (2020) who reported that yield of IWG declined, due to low grain number, few highly productive spikes, increased intra-stand competition and declined resource allocation to reproduction over time, motivating future studies focused on maintaining seed set, and thus productivity from a management perspective. The decline in the harvest index was less pronounced in the intercrop as compared to the sole crop, which may be attributed to the expected improved nitrogen supplies provided by the alfalfa root and shoot turnover resulting in a green manure (Bedoussac et al. 2015; Jensen et al. 2020). The growing season of 2018 was unusually dry and warm, resulting in severe drought effects. The hampered growth is not only attributed to physiological constraints, but also to restricted ability of the plant to make use of nutrients, due to lack of precipitation and resulting low soil moisture level. In fact, the digestate applied that year was crusting on the soil surface, seemingly inaccessible to the crops during peak of growth, but later in the season, the nutrients may have become accessible. However, alfalfa did sustain its growth also under the dry conditions in 2018, probably due to its ability to biologically to fix N_2.

3.2 Nitrogen concentration in dry matter and N accumulation

The grain N concentration of IWG varied between 2.7 and 3.5% N. Several intercrop studies of annual cereals and grain legumes have shown that intercropping increases the nitrogen/protein concentration of the intercropped cereal grain and potentially also the baking quality (Gooding et al. 2007; Hauggaard-Nielsen et al. 2008). In 2018, the N concentration of IWG grain is higher when intercropped than when grown as monocrop, while the N concentration in the IWG straw was higher in the intercrop compared to the monocrop in each individual year (Table 3). In the intercrop, the N concentration in IWG straw was higher in 2017 as compared to in 2018 and 2019, while there was no difference between years in the monocrop. The N concentration of the alfalfa biomass was stable at approximately 2.6% N throughout the 3 years (Table 3).

Higher N concentration of the harvested IWG material from intercropped conditions may have positive implications for the use of the IWG straw as forage in animal production as well as the use of the IWG grain in the food industry. Surprisingly, the N concentration in the grain and straw of IWG tends to decline with time both in the sole and the intercrop. Even though just evident as a weak tendency, it can be seen in both the monocrop and the intercrop as well as for both grain and straw components of IWG. This decline may be a result of the low-input management strategy applied to the two crops in this particular experiment. The land of the experimental site was previously used for conventional cropping with a crop rotation based on only annual crops supplied with generous nutrient additions. Thus, the first experimental year is highly influenced by carry-over effects from previous management, while the subsequent years better represent the

Table 3 The nitrogen concentration (N, %) and discrimination against ^{13}C (ΔC13, ‰) presented as means ($N = 4$) with standard errors. Different lowercase letters indicate differences between treatments (crop and year) from proc MIXED in SAS (SAS 9.4) with Kenward-Rogers method for denominator degrees of freedom and Tukey's post hoc test at the $p < 0.05$ level of significance (SAS 9.4). Abbreviations IWG, intermediate wheatgrass; LUC lucerne.

	N [%]			*ΔC13* [‰]	
	IWG grain	IWG straw	LUC total	IWG	LUC
Monocrop					
2017	3.01 ± 0.029***ab***	0.655 ± 0.083***bcd***	n. a.	20.3 ± 0.20***a***	n. a.
2018	2.69 ± 0.060***b***	0.530 ± 0.023 ***cd***	n. a.	18.4 ± 0.20***c***	n. a.
2019	2.66 ± 0.23***b***	0.474 ± 0.037***d***	n. a.	19.0 ± 0.29***b***	n. a.
Intercrop					
2017	3.45 ± 0.057***a***	1.35 ± 0.15***a***	2.46 ± 0.48***a***	20.4 ± 0.12***a***	21.5 ± 0.23***a***
2018	3.39 ± 0.073***a***	0.804 ± 0.032***b***	2.69 ± 0.18***a***	17.6 ± 0.33***d***	19.1 ± 0.20***b***
2019	3.11 ± 0.39***ab***	0.714 ± 0.078***bc***	2.58 ± 0.20***a***	19.1 ± 0.21***b***	22.2 ± 0.17***a***

targeted low-input system with limited nutrient resources. In the light of the multipurpose use of IWG, i.e. both as a grain and feed crop, intercropping is known to improve the nutrient concentration of the biomass and thus its suitability as animal feed (Favre et al. 2019) or biogas digestate usage of the straw fraction. In the context of a changing climate, in terms of the more frequent drought spells, the higher N concentration in IWG grain in the dry year of 2018 may indicate a suitable crop for climate adaptation.

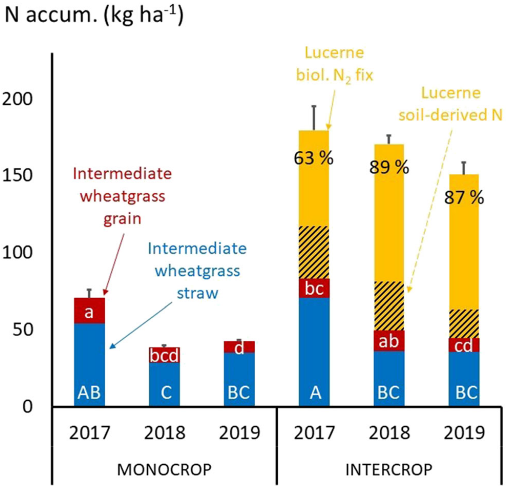


Fig. 5 The aboveground N accumulation in intermediate wheatgrass grain (red bars), intermediate wheatgrass straw (blue bars) and lucerne (yellow bars) divided into the fraction acquired from the soil and fertiliser (black-striped yellow bars) and the contribution of biological N_2 fixation (plain yellow bars). For the latter, the percentage (%Ndfa) of the N_2 fixed is given. Different lower case letters (within red bars) indicate differences in intermediate wheatgrass grain yield, and upper case letters (within blue bars) in intermediate wheatgrass straw biomass yield, from proc MIXED in SAS (SAS 9.4) with Kenward-Rogers method for denominator degrees of freedom, using univariate ANOVA with Tukey's post hoc test at the $p < 0.05$ level of significance.

The N accumulation (Fig. 5) of IWG grains was higher in 2017 than in 2018 and 2019 in the monocrop. The N accumulated in IWG grains in the intercrop was higher in 2018 than in 2019, while neither differed from 2017. Furthermore, the N accumulated in IWG grains was higher in the monocrop in 2017 than in the intercrop in 2017, while there was no difference between the cropping systems on 2018 and 2019. In the monocrop, the N accumulation of the IWG straw (Fig. 5) was higher in 2017 compared to in 2019. In the intercrop, the N accumulated in IWG straw was higher in 2017 compared to in 2018 and 2019. No differences were found between the two cropping systems within each year. The amount of N accumulated in alfalfa did not differ between years. Neither did the amount of N accumulated from the soil and fertiliser, but the amount of N derived from the atmosphere was higher in 2017 than in the 2 subsequent years (Fig. 5). Our study shows that high amounts of N in lucerne (63 ± 27, 89 ± 2.8 and 87 ± 4.3%; Fig. 5) are derived from the atmosphere and smaller amounts of N (11 to 37%) are derived from soil. The proportion of N_2 fixed from the atmosphere showed a tendency to be higher in 2018 and 2019 compared to in 2017 ($F = 4.69$ (df = 2) $p < 0.1$).

The amount of accumulated N in the studied systems is clearly connected to the amount of biomass produced, further supporting the argument above that growing conditions and production are related to the availability of resources (Hawkesford 2011), in this case nitrogen. The patterns of the N accumulation of IWG grains most probably arise from a depletion of time in the cropping systems, with the pattern more pronounced in the monocrop than in the intercrop (where the depletion is less pronounced) since legumes improve the availability of soil N (Jensen et al. 2020). Interestingly, the rather high level of accumulated N in IWG grain in the intercrop in 2018 potentially demonstrates intercropping as a management tool for production security under unexpected or deviating climate events, such as drought. The lowest values of accumulated N in IWG grains were found in the monocrop in 2019, which may indicate that the IWG crop received a suboptimal supply of N with the low-input management. However, the legume companion is expected to supplement some of the nutrient requirements with time and the tendency of increased fixation of nitrogen from the atmosphere somewhat meets this expectation. This tendency reflects the interspecies interaction in intercrops of cereals and legumes, where the cereal most often acquires a larger proportion of the soil N in comparison to its proportional abundance, which leads to increased N_2 fixation in the grain legume crop (Rodriguez et al. 2021). On the other hand, Li et al. (2019) identified a need for fertilisation to support the development of IWG in the establishment phase when intercropped with alfalfa, possibly indicating that alfalfa is too aggressive for intercropping with IWG, at least when established at the same time point. The study of Li et al. (2019) needs validation in the field, but gives clear indications that we need more knowledge on appropriate intercropping companion and establishment practices for IWG production. Surprisingly, the aboveground N accumulation in IWG straw did not differ between the monocrop and the intercrop in either of the 3 years, despite the IWG straw biomass was generally lower in the intercrop than in the sole crop. Under intercropped conditions as those in this experimental setup, the cereal is established on half of the area compared to that in the monocrop. Thus, our results clearly demonstrate the benefits of intercropping in terms of nutritional value (Favre et al. 2019; Bedoussac et al. 2015), irrespective of the end-usage of the crop.

3.3 Discrimination against ^{13}C as an indicator of water use efficiency

In the dry year of 2018 (Fig. 3), the discrimination against ^{13}C in the IWG aboveground biomass was lower in both the sole cropped and the intercropped system compared to the other years, i.e. 2017 and 2019 (Table 3). This was probably a response to drought, which has been observed in other C_3 forage grasses (Mårtensson et al. 2017), where improved water use efficiency is gained through stomatal closure (Farquhar et al. 1982; Farquhar et al. 1989). Water limitation is often closely connected to lowered availability of nutrients (Kreuzwieser and Gessler 2010) partly through the limitation of microbial activity (Borken and Matzner 2009) and hence nutrient mineralisation, but also through the restricted transport and uptake of soil water and the associated mass flow uptake of nutrients. Water limitation seems to be better met when intercropping with legumes, where IWG showed even lower discrimination against ^{13}C when intercropped with alfalfa. Some varieties of alfalfa have been described as drought resistant (Guo et al. 2005), due to their extensive and deep root system (Dolling et al. 2003; Julier et al. 2017). Indeed, the reduced discrimination under drought also occurred in alfalfa shoots, where the discrimination against ^{13}C was lower in 2018 compared to in 2017 and 2019 but this legume exhibits low stomatal closure in the early stages of drought (Durand 2007). Furthermore, it can be hypothesised that alfalfa potentially provides a shading effect on the soil surface, thus reducing the evaporation and improving the soil water status.

Under intercropped management, discrimination against ^{13}C in IWG grains was well correlated to IWG grain yield ($\rho = 0.971$, $p < 0.05$; data not shown) and IWG grain N accumulation ($\rho = 0.987$, $p < 0.05$; data not shown) in 2018. In 2019, positive correlations were found between discrimination against ^{13}C in IWG straw and both IWG straw yield ($\rho = 0.982$, $p < 0.05$; data not shown) and IWG straw N yield ($\rho = 0.961$, $p < 0.05$; data not shown) in intercrops. These results clearly demonstrate the ability of IWG to improve water use efficiency, which may lead to downregulation of the discrimination against ^{13}C to sustain photosynthesis under the dry summer of 2018 and the possibly still dry soils in 2019. These results are supported by other studies which have proven that IWG maintains relatively high water-use efficiency during the growing season (Culman et al. 2013; de Oliveira et al. 2020), which helps to mitigate water stress.

4 Conclusions

Here, we show for the first time potential benefits of the intercropping of perennial cereal crop, IWG, with alfalfa in terms of grain yield and biomass production in Scandinavia, also under a drought spell. In particular, the ability to acquire N_2 from the atmosphere to the production system under intercropped conditions over the experimental period (2017–2019) illustrates the important function of acquiring additional nitrogen from the atmosphere into this production system, especially under dry conditions to sustain photosynthesis and, thus, growth. We suggest that perennial cereal crops intercropped with legume companions could be a suitable addition to cropping systems under the expected increased frequency of drought events.

Acknowledgements Financial support for this study is gratefully acknowledged from the Crafoord Foundation. This study has also been made possible by the Swedish Infrastructure for Ecosystem Science (SITES), in this case at the Lönnstorp Research Station at Alnarp, Sweden. We would also like to thank Ryan Davidson and Karl-Erik Gustavsson for valuable assistance in experimental management, sampling and laboratory analyses. Jan-Eric Englund and Adam Flöhr are gratefully acknowledged for valuable statistical assistance.

Authors' contributions Jensen was responsible for the development and design of the SAFE. Li carried out sample preparation for laboratory analyses. Dimitrova Mårtensson carried out statistical analyses. Results were interpreted by Dimitrova Mårtensson, Li, Barreiro and Jensen. The manuscript was written by Dimitrova Mårtensson with input from Li, Barreiro and Jensen.

Funding Open access funding provided by Swedish University of Agricultural Sciences. The Crafoord Foundation 20160622

Data availability The data generated and analysed during this study are available from the corresponding author upon reasonable request.

Code availability Not applicable.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interests The authors declare no competing interests.

References

Asbjornsen H, Hernandez-Santana V, Liebman M, Bayala J, Chen J, Helmers M, Ong CK, Schulte LA (2014) Targeting perennial vegetation in agricultural landscapes for enhancing ecosystem services. Renew Agric Food Syst 29:101–125. https://doi.org/10.1017/S1742170512000385

Bedoussac L, Journet EP, Hauggaard-Nielsen H, Naudin C, Corre-Hellou G, Jensen ES, Prieur L, Justes E (2015) Ecological principles underlying the increase of productivity achieved by cereal-grain legume intercrops in organic farming. A review. Agron Sustain Dev 35:911–935. https://doi.org/10.1007/s13593-014-0277-7

Borken W, Matzner E (2009) Reappraisal of drying and wetting effects on C and N mineralization and fluxes in soils. Glob Chang Biol 15:808–824. https://doi.org/10.1111/j.1365-2486.2008.01681.x

Coplen TB (2011) Guidelines and recommended terms for expression of stable isotope-ratio and gas-ratio measurement results. Rapid Commun Mass Spectrom 25:2538–2560. https://doi.org/10.1002/rcm.5129

Crews T, Blesh J, Culman SW, Hayes RC, Jensen ES, Mack MC, Peoples MB, Schipanski ME (2016) Going where no grains have gone before: From early to mid-succession. Agric Ecosyst Environ 223:223–238. https://doi.org/10.1016/j.agee.2016.03.012

Crews TE, Carton W, Olsson L (2018) Is the future of agriculture perennial? Imperatives and opportunities to reinvent agriculture by shifting from annual monocultures to perennial polycultures. Glob Sustain 1:1–18. https://doi.org/10.1017/sus.2018.11

Culman S, Snapp SS, Ollenburger M, Basso B, Dehaan L (2013) Soil and water quality rapidly responds to the perennial grain Kernza wheatgrass. Agron J 105:735–744. https://doi.org/10.2134/agronj2012.0273

Dalal RC, Strong WM, Cooper JE, King AJ (2013) Relationship between water use and nitrogen use efficiency discerned by 13C discrimination and 15 N isotope ratio in bread wheat grown under no-till. Soil Tillage Res 128:110–118. https://doi.org/10.1016/j.still.2012.07.019

de Oliveira G, Brunsell N, Crews TE, DeHaan L, Vico G (2020) Carbon and water relations in perennial Kernza (*Thinopyrum intermedium*): an overview. Plant Sci 295:735–744. https://doi.org/10.2134/agronj2012.0273

de Oliveira G, Brunsell NA, Crews TE, DeHaan LR, Vico G (2019) Carbon and water relations in perennial Kernza (*Thinopyrum intermedium*): An overview. Plant Science 295:110279. https://doi.org/10.1016/j.plantsci.2019.110279

DeHaan L, Wang S, Larson SR, Kantarski T, Zhang X, Cattani D, Viinanen TR (2013) Current efforts to develop perennial wheat and domesticate Thinopyrum intermedium as a perennial grain. Perennial Crops for Food Security, Proceedings of the FAO Expert Workshop, Genetics and breeding: State of the Art, Gaps and Opportunities. Corpus ID: 91322649. https://landinstitute.org/wp-content/uploads/2014/11/PF_FAO14_ch06.pdf

Dick C, Cattani D, Entz MH (2018) Kernza intermediate wheatgrass (*Thinopyrum intermedium*) grain production as influenced by legume intercropping and residue management. Can J Plant Sci 98:1376–1379. https://doi.org/10.1139/cjps-2018-0146

Dolling PJ, Ward PR, Latta RA, Ryder A, Asseng S, Robertson MJ, Cocks PS, Ewing MA (2003) Rate of root growth in lucerne varies with soil type in Western Australia. Australian Society of Agronomy. "Solutions for a better environment". Proceedings of the 11th Australian Agronomy Conference, 2-6 Feb. 2003, Geelong, Victoria. ISBN 0-9750313-0-9

Duchene O, Celette F, Ryan MR, Dehaan LR, Crews TE, David C (2019) Integrating multipurpose perennial grains crops in Western European farming systems. Agric Ecosyst Environ 284:106591. https://doi.org/10.1016/j.agee.2019.106591

Duchene O, Celette F, Barreiro A, Dimitrova Mårtensson LM, Freschet GT, David C (2020) Introducing perennial grain in grain crops rotation: the role of rooting pattern in soil quality management. Agron 10:1–17. https://doi.org/10.3390/agronomy10091254

Durand JL (2007) Effects of water shortage on forage plants. Fourrages 190:181–196

FAO (2013) Perennial Crops for Food Security. Proceedings of the FAO Expert Workshop 28-30 August, 2013, Rome, Italy. ISBN 978-92-5-107998-0

Farquhar GD, Richards RA (1984) Isotopic composition of plant carbon correlates with water-use efficiency of wheat genotypes. Aust J Plant Physiol 11:539–552. https://doi.org/10.1071/PP9840539

Farquhar GD, Oleary MH, Berry JA (1982) On the relationship between carbon isotope discrimination and the inter-cellular carbon-dioxide concentration in leaves. Aust J Plant Physiol 9:121–137. https://doi.org/10.1071/PP9820121

Farquhar GD, Ehleringer JR, Hubick KT (1989) Carbon isotope discrimination and photosynthesis. Annu Rev Plant Physiol Plant Mol Biol 40:503–537. https://doi.org/10.1146/annurev.pp.40.060189.002443

Favre JR, Munoz Castiblanco T, Combs DK, Wattiaux MA, Picasso VD (2019) Forage nutritive value and predicted fiber digestibility of Kernza intermediate wheatgrass in monoculture and in mixture with red clover during the first production year. Anim Feed Sci Technol: 258. https://doi.org/10.1016/j.anifeedsci.2019.114298

Gooding MJ, Kasyanova E, Ruske R, Hauggaard-Nielsen H, Jensen ES, Dahlmann C, von Fragstein P, Dibet A, Corre-Hellou G, Crozat Y, Pristeri A, Romeo M, Monti M, Launay M (2007) Intercropping with pulses to concentrate nitrogen and sulphur in wheat. J Agric Sci 145:469–479. https://doi.org/10.1017/S0021859607007241

Gross N, Suding K, Lavorel S, Roumet C (2007) Complementarity as a mechanism of coexistence between functional groups of grasses. J Ecol 95:1296–1305. https://doi.org/10.1111/j.1365-2745.2007.01303.x

Guo ZG, Liu HX, Wang SM, Tian FP, Cheng GD (2005) Biomass, persistence and drought resistance of nine lucerne varieties in the dry environment of west China. Aust J Exp Agric 45:59–64. https://doi.org/10.1071/ea03119

Hauggaard-Nielsen H, Jørnsgaard B, Kinane J, Jensen ES (2008) Grain legume–cereal intercropping: the practical application of diversity, competition and facilitation in arable and organic cropping systems. Renew Agric Food Syst 23:3–12. https://doi.org/10.1017/S1742170507002025

Hawkesford MJ (2011) An overview of nutrient use efficiency and strategies for crop improvement. In: The molecular and physiological basis of nutrient use efficiency in crops, pp. 5-19. https://doi.org/10.1002/9780470960707

Högberg P (1997) Tansley Review No. 95 15 N natural abundance in soil-plant systems. New Phytol 137:179–203. https://doi.org/10.1046/j.1469-8137.1997.00808.x

Hunter MC, Sheaffer CC, Culman SW, Jungers JM (2020) Effects of defoliation and row spacing on intermediate wheatgrass I: Grain production. Agron J 112:1748–1763. https://doi.org/10.1002/agj2.20128

Jensen ES, Peoples MB, Boddey RM, Gresshoff PM, Hauggaard-Nielsen H, Alves BJR, Morrison MJ (2012) Legumes for mitigation of climate change and the provision of feedstock for biofuels and biorefineries. A review. Agron Sustain Dev 32:329–364. https://doi.org/10.1007/s13593-011-0056-7

Jensen ES, Carlsson G, Hauggaard-Nielsen H (2020) Intercropping of grain legumes and cereals improves the use of soil N resources and reduces the requirement for synthetic fertilizer N: a global-scale analysis. Agron Sustain Dev 40:5. https://doi.org/10.1007/s13593-020-0607-x

Johansen A, Jensen ES (1996) Transfer of N and P from intact or decomposing roots of pea to barley interconnected by an arbuscular mycorrhizal fungus. Soil Biol Biochem 28:73–81. https://doi.org/10.1016/0038-0717(95)00117-4

Julier B, Gastal F, Louarn G, Badenhausser I, Annicchiarico P, Crocq G, Emile JC (2017) Lucerne (Alfalfa) in European Cropping Systems. In: Legumes in Cropping Systems. CABI. ISBN 9781780644981

Jungers JM, Dehaan LH, Mulla DJ, Sheaffer CC, Wyse DL (2019) Reduced nitrate leaching in a perennial grain crop compared to maize in the Upper Midwest, USA. Agric Ecosyst Environ 272:63–73. https://doi.org/10.1016/j.agee.2018.11.007

Kørup K, Lærke PE, Baadsgaard H, Andersen MN, Kristensen K, Münnich C, Didion T, Jensen ES, Mårtensson L-M, Jørgensen U (2018) Biomass production and water use efficiency in perennial grasses during and after drought stress. GCB Bioenergy 10:12–27. https://doi.org/10.1111/gcbb.12464

Kreuzwieser J, Gessler A (2010) Global climate change and tree nutrition: influence of water availability. Tree Physiol 30:1221–1234. https://doi.org/10.1093/treephys/tpq055

Landis DA (2017) Designing agricultural landscapes for biodiversity-based ecosystem services. Basic Applied Ecol 18:1–12. https://doi.org/10.1016/j.baae.2016.07.005

Li S, Barreiro A, Jensen ES, Zhang Y, Dimitrova Mårtensson LM (2019) Early interspecific dynamics, dry matter production and nitrogen use in Kernza (Thinopyrum intermedium) - alfalfa (Medicago sativa) mixed intercropping. Acta Agr Scand B-SP 70:165–175. https://doi.org/10.1080/09064710.2019.1686164

Mårtensson LM, Carlsson G, Prade T, Kørup K, Lærke PE, Jensen ES (2017) Water use efficiency and shoot biomass production under water limitation is negatively correlated to the discrimination against (13)C in the C(3) grasses Dactylis glomerata, Festuca arundinacea and Phalaris arundinacea. Plant Physiol Biochem 113:1–5. https://doi.org/10.1016/j.plaphy.2017.01.021

O'Leary MH (1981) Carbon isotope fractionation in plants. Phytochem 20:553–567. https://doi.org/10.1016/0031-9422(81)85134-5

O'Leary MH (1988) Carbon Isotopes in Photosynthesis. BioSci 38:328–336. https://doi.org/10.2307/1310735

Orloff SB, Brummer EC, Shrestha A, Putnam DH (2016) Cool-season perennial grasses differ in tolerance to partial-season irrigation deficits. Agron J 108:692–700. https://doi.org/10.2134/agronj2015.0384

Park R, Epstein S (1960) Carbon isotope fractionation during photosynthesis. Geochim Cosmochim Acta 21:110–126. https://doi.org/10.1016/S0016-7037(60)80006-3

Rodriguez C, Dimitrova Mårtensson L-M, Jensen ES, Carlsson G (2021) Combining crop diversification practices can benefit cereal production in temperate climates. Agron Sustain Dev 41:1–14. https://doi.org/10.1007/s13593-021-00703-1

Roudier P, Andersson JCM, Donnelly C, Feyen L, Greuell W, Ludwig F (2016) Projections of future floods and hydrological droughts in Europe under a + 2 °C global warming. Clim Chang 135:341–355. https://doi.org/10.1007/s10584-015-1570-4

Ryan MR, Crews TE, Culman SW, DeHaan LR, Hayes RC, Jungers JM, Bakker MG (2018) Managing for Multifunctionality in Perennial Grain Crops. BioSci 68:294–304. https://doi.org/10.1093/biosci/biy014

SMHI (2019) State of knowledge and implications for adaptation and mitigation. Swedish Meteorological and Hydrological Institute. Ed: Ralf Döscher. 10.17200/Climate_Extremes_Sweden

Sprunger CD, Culman SW, Peralta AL, DuPont ST, Lennon JT, Snapp SS (2019) Perennial grain crop roots and nitrogen management shape soil food webs and soil carbon dynamics. Soil Biol Biochem 137:107573. https://doi.org/10.1016/j.soilbio.2019.107573

Tautges NE, Jungers JM, DeHaan LR, Wyse DL, Sheaffer CC (2018) Maintaining grain yields of the perennial cereal intermediate wheatgrass in monoculture v. bi-culture with alfalfa in the Upper Midwestern USA. J Agric Sci 156:758–773. https://doi.org/10.1017/S0021859618000680

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. https://doi.org/10.1007/s13593-016-0396-4

Unkovich M, Herridge D, Peoples M, Cadisch G, Boddey B, Giller K, Alves B, Chalk P (2008) Measuring plant-associated nitrogen fixation in agricultural systems. Australian Centre for International Agricultural Research (ACIAR), Canberra. ISBN 978 1 921531 26 2

Vico G, Brunsell NA (2018) Tradeoffs between water requirements and yield stability in annual vs. perennial crops. Adv Water Resour 112:189–202. https://doi.org/10.1016/j.advwatres.2017.12.014

Wagoner P, Schauer A (1990) Intermediate wheatgrass as a perennial grain crop. In: Advances in new crops - proceedings of the First National Symposium NEW CROPS, Research, Development, Economics, Indianapolis, Indiana, October 23-26, 1988. Timber Press, Portland. ISBN: 0881921661

Willey RW, Osiru DSO (1972) Studies on mixtures of maize and beans (Phaseolus vulgaris) with special reference to plant population. J Agric Sci 79:519–529. https://doi.org/10.1017/S0021859600025909

Zhang X, Sallam A, Gao L, Kantarski T, Poland J, DeHaan LR, Wyse DL, Anderson JA (2016) Establishment and optimization of genomic selection to accelerate the domestication and improvement of intermediate wheatgrass. Plant Genome 9. https://doi.org/10.3835/plantgenome2015.07.0059

III

Soil Biology and Biochemistry 200 (2025) 109621

Contents lists available at ScienceDirect

Soil Biology and Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Perennial crops shape the soil microbial community and increase the soil carbon in the upper soil layer

Shoujiao Li [a], Ana Barreiro [b], Juan Pablo Almeida [a], Thomas Prade [a], Linda-Maria Dimitrova Mårtensson [a,*]

[a] *Swedish University of Agricultural Sciences, Department of Biosystems and Technology, P.O. Box 190, SE-234 22, Lomma, Sweden*
[b] *University of Santiago de Compostela, Department of Soil Science and Agricultural Chemistry, 27002, Lugo, Spain*

ARTICLE INFO

Dataset link: Data for "Perennial crops shape the soil microbial community and increase the soil carbon in the upper soil layer" (Original data)

Keywords:
Perennial
Intermediate wheatgrass
Microbial community composition
Microbial biomass
PLFA
Soil total carbon

ABSTRACT

Soil biodiversity is threatened by intensive agriculture that relies on annual grain crop production, thus leading to a decline in soil functions and ecosystem services. Perennial grain crops have a positive impact on the soil microbial community, but the responsive microbial groups and the magnitude of their response remain uncertain. To elucidate this, we analysed soil microbial biomass and community composition, bacterial growth and soil total carbon in five crops: organic perennial intermediate wheatgrass (IWG, *Thinopyrum intermedium*, Kernza®), organic IWG-alfalfa intercrop, organic biennial grass-legume mixture, organic annual wheat or rye and conventional annual wheat. The analysis was carried out at three time points under two growing seasons at four different soil depths. Five years after establishment, IWG had greater amounts of soil total fungi and bacteria, and of arbuscular mycorrhizal (AM) fungi, saprotrophic fungi, gram-negative (G^-) and gram-positive (G^+) bacteria compared to annual wheat. Crop perenniality influenced the soil microbial community structure although precipitation, soil temperature and water content were the main drivers of the patterns of and temporal variations in the microbial community. Perennial crops, with reduced tillage and low nitrogen input management increased the proportions of fungi relative to bacteria, AM fungi to saprotrophic fungi, G^- bacteria to G^+ bacteria, and the growth rate of total bacteria. This resulted in a more active soil microbial community with higher microbial biomass than annual wheat and contributed to the increased soil total carbon storage in the 0–5 cm soil layer in a humid continental climate. The findings emphasize the importance of combining a no tillage strategy with long-term vegetation cover to increase soil quality.

1. Introduction

Soil microbial organisms are vital for agricultural production and soil health due to their significant roles in nutrient cycling and other ecosystem processes (Barrios, 2007). For example, soil arbuscular mycorrhizal (AM) fungi form symbioses with most terrestrial plants and influence plant nutrient uptake (Jeffries et al., 2003). Soil saprotrophic fungi and bacteria decompose soil organic matter and influence nutrient availability for plants (Ning et al., 2021). Symbiotic bacteria such as *Rhizobium sp.* can form nodules with legume roots and fix nitrogen from the atmosphere (Soumare et al., 2020). Furthermore, the necromass of soil microbes contributes to 24%–60% of the soil organic carbon pool and contributes to soil carbon sequestration (Deng and Liang, 2022).

Soil microbiota differs among soils depending on factors such as land use and management. Intensive agriculture with monocultures of annual crops decreases soil microbial biomass (Yan et al., 2022), reduces soil biodiversity, simplifies soil food webs, and threatens overall soil functioning (Tsiafouli et al., 2015) due to the intensive management (*e.g.* soil tillage, re-sowing, fertilizer application, chemical and mechanical weed management). As a result, annual crop production is responsible for a range of environmental problems including soil degradation and other pollution (Kopittke et al., 2019). Thus, current conventional agricultural practices for producing annual crops negatively influence soil health (Yang et al., 2020) and undermine the possibility of long-term sustainability in crop production (Cárceles Rodríguez et al., 2022).

Perennial cereal grain crops can be productive for several years without the need to be re-sown every year, significantly reducing soil disturbance otherwise resulting from tillage (Chapman et al., 2022). The year-round presence of roots, combined with the inherent capacity to grow large root biomass (Sprunger et al., 2018), perennial crops

---

* Corresponding author.
E-mail address: linda.maria.martensson@slu.se (L.-M. Dimitrova Mårtensson).

https://doi.org/10.1016/j.soilbio.2024.109621
Received 3 April 2024; Received in revised form 25 July 2024; Accepted 7 October 2024

minimize nitrate leaching (Jungers et al., 2019; Huddell et al., 2023) and emit root exudates into the soil ecosystem (Ma et al., 2022). All these factors combined result in a production system that increases soil organic matter (Kim et al., 2022), enhances water use efficiency (de Oliveira et al., 2018; Clément et al., 2022), improves soil structure (Daly et al., 2023), sustains soil fertility (Mosier et al., 2021), reduces soil erosion (Cosentino et al., 2015) and sequesters more carbon in the soil (Peixoto et al., 2022). The transition from annual to perennial cereal grain crops is therefore suggested as a way to enhance sustainability in agriculture (Duchene et al., 2020; Chapman et al., 2022; Zhang et al., 2023).

The first perennial cereal crop, intermediate wheatgrass (*Thinopyrum intermedium* (Host) Barkworth and D.R. Dewey, Kernza®) or IWG, has been selected and domesticated for grain production and forage use (Wagoner and Schaeffer, 1990; Culman et al., 2013; Bajgain et al., 2022). In the past ten years, IWG has been evaluated for its grain (Dick et al., 2018; Fernandez et al., 2020; Hunter et al., 2020a) and biomass production (Jungers et al., 2017; Favre et al., 2019; Culman et al., 2023), environmental benefits (Culman et al., 2013; DeHaan and Ismail, 2017; Rakkar et al., 2023) and economic benefits (Hunter et al., 2020b; Law et al., 2022a) under various management practices in the United States and Europe. At current stage of perennial grain crop development, the grain yield of IWG (112–1212 kg ha^{-1}, Culman et al., 2013; Law et al., 2022b) is significantly lower than conventional annual wheat (global average 4020 kg ha^{-1}, Dadrasi et al., 2023). Replacing annual wheat with IWG at this stage would cause serious loss of food production. Meanwhile, the environmental benefits provided by IWG as a pioneer perennial crop has already been proven in many studies thus IWG was suggested for production on marginal land or incorporating in the crop rotation systems to increase crop diversity (Duchene et al., 2019, 2020).

Despite the numerous advantages of perennial grain crops on soil health, relatively few studies have been conducted on the complex plant-soil-microbe interaction. The extended lifetime of perennial grains causes a prolonged interaction with the soil microbiome as compared to annual crops. How the soil microbial community responds to the increased crop longevity (*i.e.* perenniality), undisturbed upper soil layers and sustained fibrous root growth is not yet fully understood. Recent studies have provided valuable insights into how IWG and annual wheat differ regarding soil microbial community composition (Sprunger et al., 2019; Duchene et al., 2020; Mckenna et al., 2020), microbial biomass (Audu et al., 2022; Rakkar et al., 2023; Taylor et al., 2024) and the related soil carbon storage capacity (Sprunger et al., 2019; Audu et al., 2022; Taylor et al., 2023), but they have not reached a consistent conclusion regarding which is the dominating microbial community. For example, Sprunger et al. (2019) reported that IWG increased bacterial and nematode diversity and richness under certain nitrogen fertilizer levels 4 years after planting IWG. Duchene et al. (2020) found that fungal abundance, but not bacterial abundance, increased in IWG cropping in the first and second spring seasons after planting. Rakkar et al. (2023) found that perennial cropping systems improved soil microbial biomass and other soil health parameters more than annual systems at the 0–15 cm soil depth. Audu et al. (2022) reported that two-year-old IWG systems had higher microbial biomass, microbial activities and soil organic carbon compared to annual wheat at the 30–60 cm soil depth but not the 0–30 cm soil depth.

Soil depth has a great impact on soil microbial communities because unequal plant roots distribution and resource availability tends to be higher at the soil surface and declines with depth (Hao et al., 2021). Soil microbial communities are also greatly influenced by seasonal dynamics (Kramer et al., 2013; Contosta et al., 2015) since temperature and moisture are important determinants of microbial activities (Brockett et al., 2012). Furthermore, the magnitude of the response of soil microbial communities and soil health improvements due to perennial grain crops depends on the specific soil type and local climate (Rakkar et al., 2023). With the growing interest in perennial grain agriculture in Europe, it is important to evaluate how perennial grain crop production influences soil microbial biomass, communities, and soil parameters in the Scandinavian climate.

The aim of this study was to better understand how crop perenniality affects soil microbial biomass and communities. More specifically, the objectives were to investigate whether longer-duration perennial crops increase microbial diversity and abundance, and whether prolonged root development in perennial crops and reduced soil disturbance significantly contribute to enhanced soil carbon storage. Therefore, we compared soil microbial community composition, biomass, bacterial growth, and soil total carbon with regard to organic perennial cropping (IWG sole crop, IWG-alfalfa intercropping), organic biennial ley cropping, and organic and conventional annual cropping (wheat or rye) for two growing seasons at a 0–40 cm soil depth. We hypothesised that crops with high levels of perenniality would host a more diverse and more abundant soil microbial community and accumulate more carbon relative to crops with low levels of perenniality.

2. Material and methods

2.1. *Experimental site*

The experiment was conducted at the Swedish Infrastructure for Ecosystem Science's (SITES) Agroecological Field Experiment (SAFE) field located at the SITES Lönnstorp Research Station, SLU, in Alnarp (55.65° N, 13.06° E) in a region with a humid continental climate (Fig. 1). The mean annual precipitation (from 2015 to 2022) was 533 mm and the daily average temperature was 10 °C. The soil type is a sandy loam soil with 67% sand and 18% clay; the basic physical and chemical properties of the soil were reported by Li et al. (2020). The SAFE was established in 2016 and is replicated in four fully randomized blocks with each cropping system represented in every block. Within SAFE, five crops were studied: organic perennial intermediate wheatgrass (IWG, Thinopyrum intermedium, Kernza®), organic IWG-alfalfa intercrop, organic biennial grass-legume mixture, organic annual wheat or rye, and conventional annual wheat.

2.2. *Crop selection and sampling*

To study the effects of crop perenniality on soil microbes and carbon, we selected five crops from three SAFE crop system components based on their different lifespan: (1) organic IWG sole crop (perenniality level five), (2) organic IWG-alfalfa intercropping (perenniality level five) (3) ley from the organic rotation (perenniality level two), (4) rye or wheat from the organic rotation (perenniality level one), and (5) wheat from the conventional rotation (perenniality level one). To study the seasonal

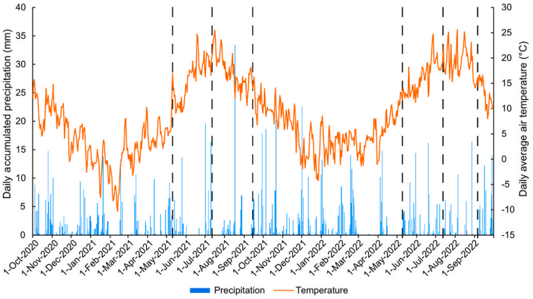


Fig. 1. The daily accumulated precipitation (mm) and average daily air (20 cm aboveground) temperature from October 1, 2020 to September 30, 2022. Dashed vertical lines indicate the sampling dates. The precipitation and air temperature data were collected by the in situ automatic weather station at the SITES Lönnstorp Research Station and retrieved from the SITES data portal (https://data.fieldsites.se/portal/).

dynamics of soil microbes, soil samples were collected at the beginning, peak and end of crop growing season; *i.e.* May, July and September in Sweden, as well as for two consecutive years (2021, 2022) for improving the reliability and repeatability of the results. Rye was part of the SAFE organic rotation system in 2021, but it was rotated out and replaced by wheat in 2022; therefore, the rye field was sampled in 2021 while the wheat field was sampled in 2022. No sampling in ley in May 2021 due to planning defects.

The IWG seeds were accessed from the Cycle 3 germplasm of the perennial grain breeding program at The Land Institute of Salinas, Kansas, USA (Zhang et al., 2016). Both IWG and IWG-alfalfa intercrops were sown with a row spacing of 25 cm, and the IWG-alfalfa intercrops were sown in separate alternate rows. The biennial crop ley is a legume-grass mixture which consists of 15% tall fescue (*Festuca arundinacea*), 10% red clover (*Trifolium pretense*), 5% white clover (*Trifolium repens*), 20% alfalfa (*Medicago sativa*), 30% timothy (*Phleum pratense*) and 20% ryegrass (*Lolium*). The planting and field management activities from 2016 to 2019 for perennial cropping systems have been previously described in detail (Audu et al., 2022; Dimitrova Mårtensson et al., 2022). The agronomic management activities, which include sowing, fertilizing, and herbicide or pesticide application in all cropping systems, are summarized in Table S1.

The plant material was sampled from one duplicate subplot (0.25 m^2) in each experimental plot at four randomized blocks on May 10, 2021, 12–13 July 2021, 14–15 September 2021, May 9, 2022, 12–13 July 2022, 5–6 September 2022 (*i.e.* five and six years after IWG planting). After collecting plant samples, soil samples were collected with a soil auger (2.5 cm diameter) at four corners of each sub-plot at 0–5, 5–15, 15–30, and 30–40 cm soil layers. The four soil augers were thoroughly mixed to obtain a composite sample per plot at each soil layer. After sampling, the soil samples were stored immediately in cool boxes and transported to the laboratory. The soil samples were sieved (through 2 mm) for homogenization within 24 h. A 50 g subsample of soil was stored in a −20 °C freezer and freeze-dried later for the microbial analyses. A 200 g subsample of soil was air-dried for pH, total carbon and nitrogen concentration analyses, and another 100 g subsample of soil was stored at 4 °C for soil bacterial growth rate and soil water content analyses.

2.3. Microbial abundance and structure

The phospholipid fatty acid (PLFA) from cell membranes varies in carbon chain length, saturation and branching in different microorganisms thus can be used as biomarkers for microbial community structure and metabolic activity in environmental studies (Willers et al., 2015). The PLFA method was chosen for the current study because PLFAs degrade rapidly after cell death and can identify living microbial biomass and is more sensitive in detecting shifts in the microbial community compared to DNA/RNA based methods, although it cannot provide detailed species composition or phylogenetic resolution when used on its own (Ramsey et al., 2006; Willers et al., 2015). Genetic analysis was not carried out in current study.

The PLFA and neutral lipids fatty acid (NLFA) analyses are based on the single-phase extraction of lipids described by Bligh and Dyer (1959) and Frostegård et al. (1993). The lipids were extracted from 2 g freeze-dried soil samples in a chloroform:methanol:citrate buffer mixture (1:2:0.8 v/v/v, pH = 4). The soil sample and mixture were vortexed and extracted at room temperature for 2 h. Then the extracts were split into two phases by adding chloroform and citrate buffer (pH = 4), and the phase containing lipid was dried under a stream of nitrogen gas. The lipid material was fractionated on a pre-packed silica column (Agilent Bond Elut, LRC, 10 ml, 40 μm) into neutral lipids, glycolipids and phospholipids by eluting with chloroform, acetone and methanol, respectively. An internal standard methyl nonadecanoate (19:0) was added to the phospholipid and neutral lipid fractions for fatty acid quantification. The samples were then methylated using a mild alkaline methanolysis to produce fatty acid methyl esters, which were then separated and quantified by gas chromatograph (polar column) with a flame ionisation detector (GC-17A, Shimadzu). The peak identification of different PLFAs and NLFAs were based on the retention times of external fatty acid methyl ester standards. Future studies should add a known amount of phospholipid, such as di19:0 PC, in the soil to serve as a recovery standard prior to extraction for quality control.

In total, 26 different fatty acids were identified in this study based on the relative retention time, and 13 of them were considered to be of bacterial origin (i15:0, a15:0, 15:0, i16:0, 16:1ω9, 16:1ω7t, 17:1ω8, i17:0, a17.0, 17:0, cy17:0, 18:1ω7, and cy19:0); the sum of these 13 PLFA was used as an indicator of total bacteria biomass (Barreiro et al., 2015). Three fatty acids were identified and considered to be of fungal origin: PLFA 18:2ω6 is an indicator for saprotrophic fungi, PLFA 18:1ω9 is a general fungal indicator and PLFA 16:1ω5 indicates AM fungi in the soil; the sum of these 3 PLFAs was used as an index of fungal biomass (Kaiser et al., 2010; Barreiro et al., 2022). Both PLFA 16:1ω5 and NLFA 16:1ω5 were used as biomarkers for AM fungi (Lehman et al., 2012; Vestberg et al., 2012; Kundel et al., 2020). The ratio between NLFA 16:1ω5 and PLFA 16:1ω5 was used to indicate the structure and abundance of AM fungi in the soil (Olsson et al., 1997; Vestberg et al., 2012). Furthermore, the G$^-$ bacteria was indicated by PLFAs 16:1ω7c, cy17:0, 18:1ω7, and cy19:0, G$^+$ bacteria was indicated by PLFAs i14:0, i15:0, i16:0, and 10Me18, and Actinobacteria was indicated by 10Me16, 10Me17, and 10Me18.

The total PLFA concentration was used as an indicator for total microbial biomass in soil, and soil microbial biomass carbon was estimated by multiplying the total PLFA concentration by a factor of 5.8 (Joergensen and Emmerling, 2006). The microbial biomass was calculated per gram of organic matter (Frostegård and Bååth, 1996). The ratio between fungi indicator PLFA abundance and bacterial indicator PLFA abundance was used to indicate the proportion of fungi relative to bacteria in the microbial community. The ratio between PLFA16:1ω5 and PLFA 18:2ω6 was used to indicate the proportion of AM fungi relative to saprotrophic fungi, and the ratio between G$^-$ bacteria and G$^+$ bacteria was used to indicate the proportion of G$^-$ bacteria relative to G$^+$ bacteria.

2.4. Bacterial activity

Bacterial activity is indicated by growth rate (*i.e.* protein synthesis rate), which was estimated by the incorporation of radiolabelled leucine into protein (Bååth, 1994; Söderberg and Bååth, 1998; Bååth et al., 2001). In brief, 1 g of fresh soil was added to 20 ml of distilled water to create the bacteria suspension, and then 1.5 ml of the bacterial suspension was incubated with 2 μl ^{3}H-labelled leucine (1 mCi 37 MBq, PerkinElmer) for 2 h at room temperature. The incubation was terminated by adding 75 μl 100% Trichloroacetic acid. After centrifugation and the removal of the supernatant, the pellet was washed with 1.5 ml 80% ethanol and 1.5 ml 5% trichloroacetic acid. The pellet was re-suspended in 200 μl 1 M NaOH and heated at 90 °C for 1 h. A 1 ml scintillator cocktail (Sigma-Aldrich, United Kingdom) was then added and the radioactivity was counted in a scintillation counter (Hidex 300 SL).

2.5. Soil physico-chemical properties

The soil total carbon and nitrogen concentration were analysed by a FLASH 2000 Organic Elemental Analyzer (Thermo Scientific) using air-dried and milled (<1 mm) soil samples. Soil organic matter (SOM) concentration was determined as soil loss on ignition at 550 °C for 3 h (Hoogsteen et al., 2015) and calculated according to Moebius-Clune et al. (2016). Soil organic carbon (SOC) concentration was estimated from soil organic matter using an SOC-to-SOM conversion factor of 0.58 g SOC/g SOM (Heikkinen et al., 2021). Apparent soil bulk density was estimated from the soil organic carbon concentration using pedotransfer functions (Kätterer et al., 2006). Soil total carbon mass per unit soil area was calculated as the product of soil total carbon concentration, apparent soil bulk density, and soil thickness at fixed soil depths (Ellert et al., 2001). Soil water content was calculated as the percentage of fresh soil weight loss after drying in an oven at 105 °C for 24 h. Soil temperature was monitored by soil temperature and moisture sensors (CS655, Campbell Scientific) installed 10 cm deep in the experimental fields from 10 May to July 20, 2021 and from 16 May to September 23, 2022. The soil temperature data for September 14, 2021 and May 9, 2022 were obtained from the weather station at the research station.

2.6. Statistical analysis

Multivariate analyses were used to show the patterns of PLFA data and to analyse with explanatory variables. The relative abundance of individual PLFAs (the percentage of each fatty acid's peak area within the total fatty acid's peak area) was used to coordinate data for multivariate analyses. The constrained ordination method called Partial Redundancy Analysis (pRDA) was used to explain the variation in the soil PLFA profile due to environmental variables after accounting for the variation explained by blocks. Stepwise Regression Forward Selection (based on 999 permutations) was conducted in R to select the relevant explanatory variables, and Holm correction was used to correct the significance level. Based on the forward selection, only significant (adjusted $p < 0.05$, Holm correction) constrained explanatory variables were included in the pRDA model ($p < 0.001$). Nine explanatory variables—precipitation, soil depth, sampling time (May 2021, July 2021, and September 2022), crop perenniality, soil water content, soil temperature and soil total carbon concentration—were selected by forward selection based on the level of significance to conduct the pRDA. The explanatory variable precipitation was defined as the accumulated rainfall during the two weeks prior to the sampling dates. The explanatory variable soil temperature was defined as the daily average soil temperature on the sampling day. The significance of each explanatory variable was tested individually and shown to be significant ($p = 0.026$ each). The first six canonical axes resulting from the pRDA were also statistically significant ($p = 0.001$). The significance of the differences between different cropping systems was tested by PERMANOVA analysis, the ADONIS test and multilevel pairwise comparison using the vegan package (Oksanen et al., 2013).

A randomized complete block design was selected for the experiment, with three treatment factors—cropping system, soil depth and sampling time—and repeated measurements of experiment units (block: cropping systems interaction) at different sampling times. The effects of cropping system, soil depth, sampling time and their interactions on the estimated microbial biomass and ratios, bacterial growth, total microbial biomass carbon and soil total carbon (concentration and mass) were tested by analysis of variance (ANOVA). A three-way ANOVA (Type III analysis of variance with Kenward-Roger's method) was used to test the effects of cropping system, soil depth and sampling time for the complete and balanced data set consisting of perennial IWG sole cropping, perennial IWG-alfalfa intercropping, biennial ley cropping and conventional annual wheat cropping at all soil depths during the sampling times of July 2021, September 2021, May 2022, July 2022, and September 2022. A two-way ANOVA was used to test the effects of cropping system and soil depth for the complete and balanced data set in May 2021. Specifically, the effects of the treatment were fitted in a linear mixed-effects model and analysed by ANOVA. Models were created using the "lme4" package, with cropping system, soil depth, and sampling time treated as fixed effects for the three-way ANOVA (or cropping system and soil depth treated as fixed effects for the two-way ANOVA). The blocks and experiment units (block:cropping systems interaction) are treated as random effects since the observations from the same experiment unit may be correlated. The outliers in AM fungi to saprotrophic fungi ratio data were replaced by the median values of other replicates. Assumptions of normally distributed residual errors and homogeneity of variance were checked by plotting residuals against fitted values and QQ plots. Box-Cox transformation was used for all the parameters to fulfil the assumptions of normality. The estimated marginal means of these variables calculated with the "emmeans" package were reported as treatment means. Pairwise comparisons of means were conducted with Tukey's method adjustment for multiple comparisons. All the statistical tests use $\alpha = 0.05$ as the significance level of effects. The Kendall correlation was used to analyse the correlation between soil microbial biomass carbon and soil total carbon concentration. All analyses were performed using R statistical software (R studio, version 4.2.0).

3. Results

3.1. Soil microbial community composition and main drivers

The pRDA model (adjusted $R^2 = 0.47$, $p = 0.001$) shows that 48.1% of the variation in soil PLFA profiles can be explained by the constrained explanatory variables of precipitation, soil depth, sampling time, crop perenniality, soil water content, soil temperature and soil total carbon concentration, with their magnitude of influence (indicated by the increases of adjusted R^2) corresponding to the order presented above (Fig. 2). Furthermore, 1.59% of the variation can be explained by the conditioned variable (block), while 50.3% of the variation was left unexplained.

Most of the variation was aggregated on the first constrained axis RDA1 (32.6% of the variance) along which soil PLFAs were associated with either high soil temperature, high precipitation and high soil water

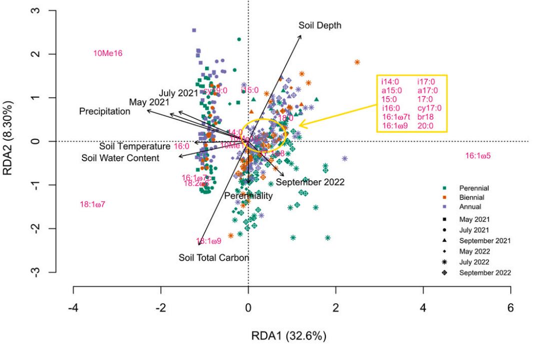


Fig. 2. Partial redundancy analysis (pRDA) of soil microbial PLFA profiles in 2021 and 2022. The name of fatty acids consist of total numbe of carbon atoms: number of double bounds, followed by the position of the double bound from the methyl end of the molecule. Cis and trans configurations are indicated by c and t. The prefixes a and i indicate anteiso- and iso-branching; br indicates unknown methyl branching position; cy indicated to cyclopropane fatty acids; and 10Me indicates a methyl group on the tenth carbon atom from the carboxyl end of the molecule. Colours indicate crop perenniality: green indicates perennial crops that were five years old including both intermediate wheatgrass sole crop (IWG) and the intercrop of IWG with alfalfa, orange indicates biennial crops that were two years old including only ley crop, and purple indicates annual crops that were one year old including organic winter wheat, rye and conventional winter wheat. Symbols indicate sampling time points, with squares indicating May 2021, circles July 2021, triangles September 2021, rhombuses May 2022, stars July 2022 and diamonds with plus sign September 2022. Arrows indicate the direction in which the gradient of the environmental variable was greatest, and the length of arrow indicates the strength of the correlation between the environmental variables and the microbes. Only significant variables based on stepwise regression forward selection ($p < 0.05$; based on 999 permutations) are displayed. The first constrained axis RDA1 and the second constrained axis RDA2 have the largest eigenvalues, and their contribution to the variance is the largest.

content in May and July 2021 (negative RDA1 scores), or low soil temperature, low precipitation, and low water content in September 2022 (positive RDA1 scores) (Fig. 2). Statistically, the main effect of the explanatory variables for May 2021 ($F = 80.5$, $p = 0.001$), July 2021 ($F = 127$, $p = 0.001$) and September 2022 ($F = 15.6$, $p = 0.001$) on soil microbial PLFAs was significant, while the main effect of the explanatory variables for September 2021, May 2022 and July 2021 was non-significant (according to the forward selection). Therefore, the latter three explanatory variables were not presented as arrows in the pRDA plot (Fig. 2).

The soil microbial community in May 2021 was significantly different from that of July 2021 and September 2022 (Pairwise Comparison, $p = 0.001$). The soil microbial community in July 2021 was also significantly different from that of September 2022 (Pairwise Comparison, $p = 0.001$). Specifically, the PLFA indicators for Actinobacteria (10Me16, 10Me17, 10Me18), G^- bacteria (16:1ω7c, 18:1ω7, cy19:0), saprotrophic fungi (18:2ω6) and general bacteria (16:0) were more abundant under high soil temperature and high precipitation conditions of May and July 2021 (Fig. 2); *i.e.* respectively, 37.8 and 49.6 mm accumulated rainfall in two weeks prior to sampling, 17.0 and 20.7 °C average air temperature (Figs. 1), and 23.6 and 17.9 °C average soil temperatures on the sampling days (May 10 and July 12, 2021). On the other hand, the PLFA indicators for AM fungi (16:1ω5), general bacteria (15:0, 16:1ω7t, 16:1ω9, a17:0, i17:0, 17:1ω8) and G^+ bacteria (i14:0, i16:0) were more abundant under low soil temperature and low precipitation conditions of September 2022 (Fig. 2); *i.e.* 16.6 mm accumulated rainfall in two weeks prior to sampling, 16.2 °C average air temperature (Figs. 1), and 19.5 °C average soil temperature on the sampling day (September 5, 2022).

The crop perenniality gradients associated with the second constrained axis RDA2 (8.30% of the variance) differentiated the perennial crops, which were characterized by a higher soil total carbon concentration in the upper soil layers (negative RDA2 scores), from the annual crops, which were characterized by a lower soil total carbon concentration in the deeper soil layers (positive RDA2 scores) (Fig. 2). Statistically, the main effect of crop perenniality on the soil microbial community composition was significant (PERMANOVA, Adonis2, $F = 18.3$, $p = 0.001$). There were separations of soil microbial PLFAs among the centres of three crop perenniality clusters (Fig. S1). The soil microbial community under perennial crops was significantly different from that under annual crops (Pairwise Comparison, $p = 0.001$) and biennial crops (Pairwise Comparison, $p = 0.005$). The soil microbial community under biennial crops was also significantly different from annual crops (Pairwise Comparison, $p = 0.03$). Specifically, the PLFA indicators for general fungi (18:1ω9), saprotrophic fungi (18:2ω6), G^- bacteria (16:1ω7c, 18:1ω7) and AM fungi (16:1ω5) were more abundant when there was a higher degree of perenniality and higher soil total carbon, while PLFA indicators for G^+ bacterial (i15:0), G^- bacteria (cy19:0), and Actinobacteria (10Me16) were more abundant when there was a lower degree of perenniality and low soil total carbon. Overall, both fungal (18:2ω6, 18:1ω9, 16:1ω5) and bacterial (*e.g.* 16:1ω7c, 18:1ω7) PLFA indicators were abundant in perennial and biennial cropping systems, while only bacterial (*e.g.* 15:0, i17:0, i15:0, cy19:0) PLFA indicators were abundant in annual wheat cropping, indicating a shift in soil microbial community composition towards a more fungi-abundant community with perennial cropping.

Soil depth also contributed to the variations associated with the RDA2 axis, and it influences the pattern of soil microbial community ordination significantly (PERMANOVA, Adonis2, $F = 23.0$, $p = 0.001$) (Fig. 2). Different soil depths had significantly different soil microbial community compositions (Pairwise Comparison, $p = 0.001$). The PLFA indicators for saprotrophic fungi (18:2ω6), fungi (18:1ω9), G^- bacteria (18:1ω7, 16:1ω7c), and Actinobacteria (10Me17) were more abundant in the wet upper soil layers 0–5 cm and 5–15 cm (Fig. 2) with high soil water content (10.6% and 10.0%, respectively). The PLFA indicators for general bacteria (*e.g.* 16:1ω9, a17:0, a15:0, 15:0, 17:0) and G^+ bacteria (i14:0, i15:0, i16:0) were more abundant in the dry lower soil layers at 15–30 cm and 30–40 cm with low soil water content (8.90% and 7.85%, respectively).

3.2. *Microbial biomass, structure and activity in different cropping systems*

3.2.1. *Perennial IWG sole crop vs. conventional annual wheat*

Crops with different levels of perenniality had different amounts of soil total microbes, total fungi, total bacteria, AM fungi, saprotrophic fungi, G^- bacteria, G^+ bacteria, and Actinobacteria, as well as a different fungi:bacteria ratio, AM fungi:saprotrophic fungi ratio, and G^- bacteria:G^+ bacteria ratio; the magnitude of these differences depended on the soil depth and sampling time point (Fig. 3; Fig. 4, Table S2, Table S3). The high perenniality IWG sole crop had significantly higher ($p < 0.05$) total PLFA biomass than low perenniality conventional annual wheat at a soil depth of 0–30 cm in May 2021, 0–40 cm in May 2022, 0–15 cm in July 2021 and July 2022, 0–5 cm in September 2021, and 5–15 cm in September 2022 (Fig. S2). The IWG sole crop had significantly ($p < 0.05$) higher estimated AM fungi (NLFA 16:1ω5) biomass than conventional wheat at almost all soil depths and sampling time points except for September 2022, during which it was observed only in lower soil layers at 15–40 cm (Fig. 3). The estimated saprotrophic fungi (PLFA 18:2ω6) biomass was higher as well under the perennial IWG sole crop ($p < 0.05$) than under conventional annual wheat at all soil depths in May 2021 (Fig. 3), while in July 2021, May 2022 and July 2022, it was mainly higher in the upper soil layer at 0–5 cm. For total fungi, total bacteria and G^- bacteria, the biomass were significantly higher ($p < 0.05$) in the IWG sole crop than the conventional annual wheat crop mainly in the upper soil layers at 0–5 cm and/or 5–15 cm except for May 2021 and May 2022, during which the values of these microbial group indicators were higher in IWG at almost all soil depths (Fig. 3). Similarly, the amount of G^+ bacteria was significantly higher ($p < 0.05$) in the perennial IWG sole crop than in the conventional annual wheat crop in the upper soil layers, specifically at 0–15 cm in May 2021 and July 2021 and at 0–5 cm and 15–30 cm in May 2022 (Fig. 3). On the other hand, Actinobacteria were more abundant in the perennial IWG sole crop than in the conventional annual wheat crop but in the deeper layers at 5–30 cm in May 2021, 5–15 cm in July 2021 and 30–40 cm in July 2022 (Fig. 3).

Regarding bacterial growth, the perennial IWG sole crop had significantly higher ($p < 0.05$) values than the conventional annual wheat crop at almost all soil depths in May 2021, July 2021 and September 2021, and in shallow soil layers 0–5 cm in May 2022 and in 15–30 cm in September 2022 (Fig. 5). The perennial IWG sole crop had a significantly higher ($p < 0.05$) microbial biomass carbon concentration than conventional annual wheat crop at 0–30 cm in May 2021 and July 2021, 0–15 cm in September 2021, July 2021 and September 2022, and 0–5 cm in May 2022 (Fig. 6).

Finally, the different ratios were also significantly higher ($p < 0.05$) for the perennial IWG sole crop compared with the annual wheat at different soil depths. The fungi:bacteria ratio was higher at soil depths of 0–5 cm and 30–40 cm in May 2021, 30–40 cm in July 2021 and May 2022, and 15–30 cm in September 2022 (Fig. 4). The AM fungi:saprotrophic fungi ratio was higher at a lower soil depth of 15–40 cm in May 2021 and July 2022, and at almost all soil depths in July 2021 and September 2022 (Fig. 4). The G^- bacteria:G^+ bacteria ratio was higher at 0–15 cm in May 2021 and at almost all depths in July 2022 (Fig. 4).

3.2.2. *Perennial IWG sole crop vs. biennial ley crop*

The differences in the estimated biomass of AM fungi (NLFA 16:1ω5), saprotrophic fungi (PLFA 18:2ω6), total fungi, total bacteria, G^- bacteria, G^+ bacteria, and Actinobacteria, as well as the fungi:bacteria ratio, AM fungi:saprotrophic fungi ratio, and G^- bacteria:G^+ bacteria ratio between the perennial IWG sole crop and biennial ley crop were not significant in most combinations of soil depths and sampling time points

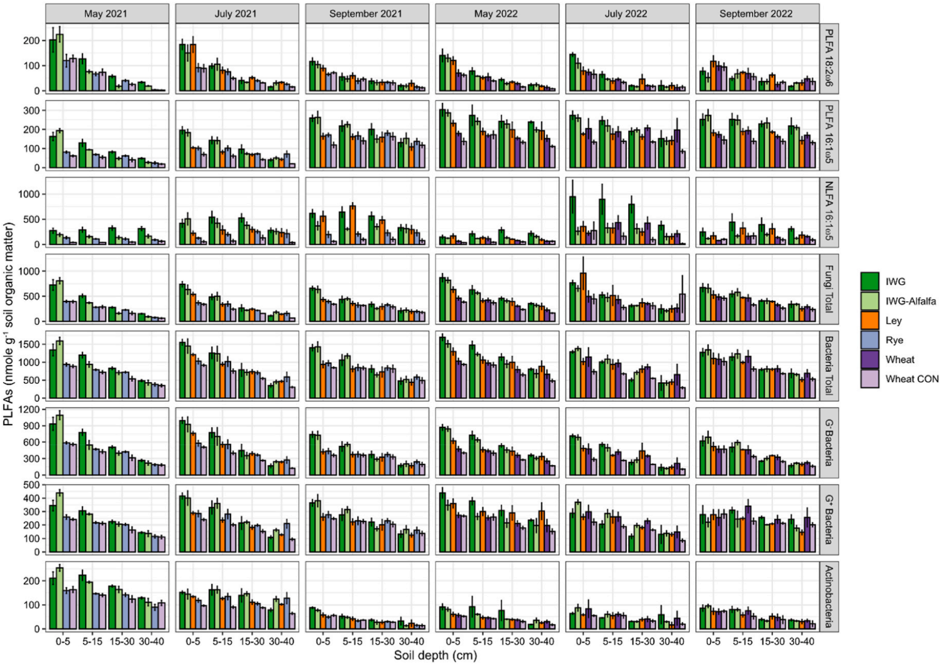


Fig. 3. Quantification of soil microbial PLFA profiles at four soil depths in 2021 and 2022. Colours indicate a specific crop: dark green indicates the perennial Intermediate Wheatgrass (IWG) sole crop under organic management, light green indicates intercropped IWG with alfalfa under organic management, orange indicates the biennial ley crop under organic management, light blue indicates annual rye under organic management, dark purple indicates annual wheat under organic management, and light purple indicates annual wheat under conventional (CON) management.

(Fig. 3; Fig 4, Table S2, Table S3). In several circumstances, the high perenniality IWG sole crop had a significantly higher ($p < 0.05$) estimated biomass of these microbial groups than the lower perenniality biennial ley crop. For example, the perennial IWG sole crop had a higher ($p < 0.05$) estimated AM fungi (NLFA 16:1ω5) biomass than the biennial ley crop at all soil depths in July 2022 (Fig. 3), a higher ($p < 0.05$) estimated saprotrophic fungi (PLFA 18:2ω6) biomass at 0–5 cm in July 2022, a higher ($p < 0.05$) estimated total bacterial biomass and G^- bacterial biomass at 0–5 cm in September 2021 and at 5–15 cm in May 2022, and a higher G^+ bacterial biomass at 30–40 cm in September 2022 (Fig. 3). However, the perennial IWG sole crop had a significantly ($p < 0.05$) lower estimated total bacterial biomass and G^- bacterial biomass than the biennial ley crop at 15–30 cm in July 2022 (Fig. 3), and a lower soil total carbon concentration and mass at 15–30 cm in September 2022 (Fig. 6; Fig. S3).

Bacterial growth was higher ($p < 0.05$) for the perennial IWG sole crop than the biennial ley crop at all soil depths in September 2022 and at 0–5 cm in May 2022 (Fig. 5). The perennial IWG sole crop had a higher ($p < 0.05$) total microbial biomass than the biennial ley crop only at 5–15 cm in May 2022 (Fig. S2), a higher ($p < 0.05$) microbial biomass carbon concentration at 0–5 cm in July 2021, September 2021 and September 2022 (Fig. 6), and a higher ($p < 0.05$) soil total carbon concentration at 0–5 cm at all sampling time points (Fig. 6).

The ratios were generally higher for the perennial IWG sole crop than the biennial ley crop. The fungi:bacteria ratio was higher at 30–40 cm in May 2022 and at 5–30 cm in July 2022 (Fig. 4). The AM fungi:saprotrophic fungi ratio was higher at 15–40 cm in July 2021 and July 2022, and at 0–5 cm in September 2022 (Fig. 4). The G^- bacteria:G^+ bacteria ratio was higher at 5–15 cm in July 2022, but lower at 15–40 cm in September 2022 (Fig. 4).

3.2.3. *Biennial ley crop vs. conventional annual wheat crop*

The biennial ley crop had a similar amount of soil total microbes, total fungi, total bacteria, AM fungi, saprotrophic fungi, G^- bacteria, G^+ bacteria, and Actinobacteria, and a similar fungi:bacteria ratio, AM fungi:saprotrophic fungi ratio, and G^- bacteria:G^+ bacteria ratio compared to the conventional annual wheat crop in most combinations of soil depths and sampling time points (Fig. 3; Fig. 4, Table S2, Table S3). Under several conditions, the biennial ley crop had a significantly higher ($p < 0.05$) biomass of these microbial groups than the conventional annual wheat crop. For example, the biennial ley crop had a higher ($p < 0.05$) amount of AM fungi (NLFA 16:1ω5) than conventional annual wheat crop at almost all soil depths in July 2021 and September 2021 (Fig. 3). The biennial ley crop had a higher ($p < 0.05$) saprotrophic fungal (PLFA 18:2ω6) and total fungi biomass than conventional annual wheat at 0–5 cm in May 2022 and at 30–40 cm in July 2021 and May 2022 (Fig. 3). The amounts of total bacteria and G^+ bacteria were higher for the ley crop than the conventional annual wheat crop at 15–40 cm in May 2022, while the amount of G^- bacteria was higher at 0–5 cm and 30–40 cm in May 2022, and at 0–5 cm and 15–30 cm in July 2022 (Fig. 3).

Bacterial growth was less for the biennial ley crop than the conventional annual wheat crop, but only at 0–5 cm in September 2022 (Fig. 5). The biennial ley crop had a higher ($p < 0.05$) total microbial biomass than the conventional annual wheat crop at 0–5 cm in July 2022 (Fig. S2), a higher microbial biomass carbon concentration at 0–5 cm in July 2022 and at 0–5 and 15–30 cm in July 2021 (Fig. 6), and a higher soil total carbon concentration at 15–30 cm in September 2022 (Fig. 6).

For the ratios, the biennial ley crop had a higher ($p < 0.05$) fungi:bacteria ratio than the conventional annual wheat crop at 30–40 cm in

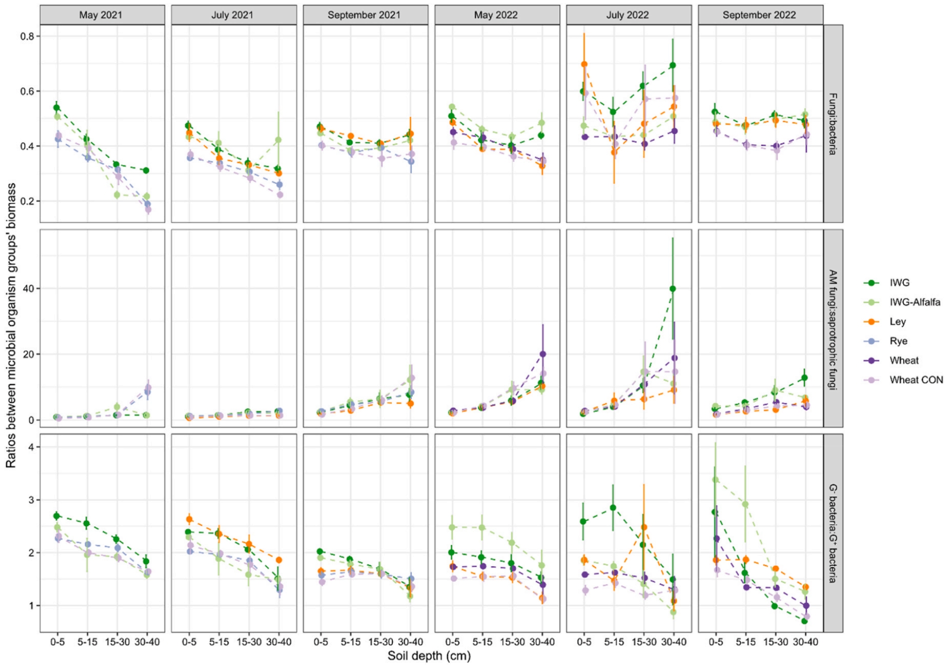


Fig. 4. Ratios of total fungi to total bacteria, arbuscular mycorrhizal (AM) fungi to saprotrophic fungi, and gram negative (G^-) to gram positive (G^+) bacteria at four soil depths in 2021 and 2022. Colours indicate a specific crop: dark green indicates the perennial intermediate wheatgrass (IWG) sole crop under organic management, light green indicates intercropped IWG with alfalfa under organic management, orange indicates the biennial ley crop under organic management, light blue indicates annual rye under organic management, dark purple indicates annual wheat under organic management, and light purple indicates annual wheat under conventional (CON) management.

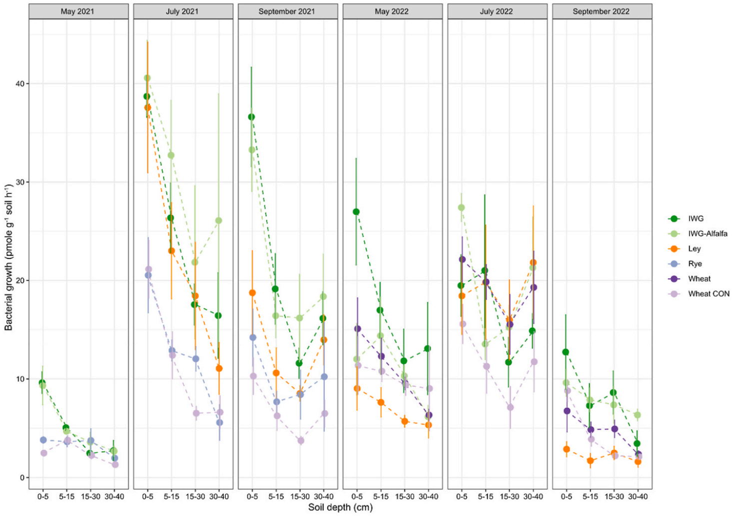


Fig. 5. Bacteria growth at four soil depths in 2021 and 2022. Colours indicate a specific crop: dark green indicates the perennial intermediate wheatgrass (IWG) sole crop under organic management, light green indicates intercropped IWG with alfalfa under organic management, orange indicates the biennial ley crop under organic management, light blue indicates annual rye under organic management, dark purple indicates annual wheat under organic management, and light purple indicates annual wheat under conventional (CON) management.

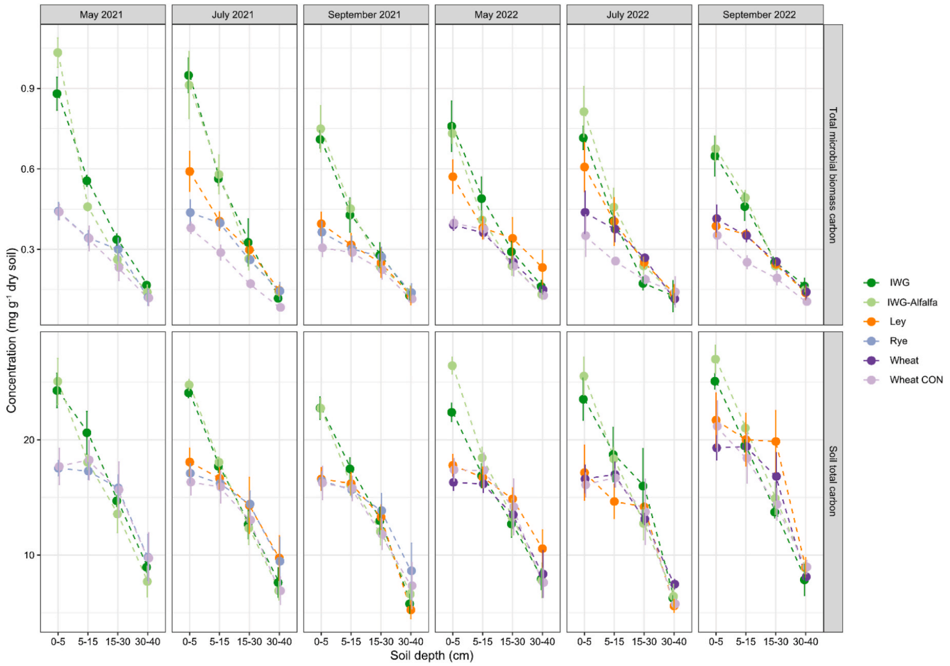


Fig. 6. Soil total microbial biomass carbon concentration and soil total carbon concentration at four soil depths at four soil depths in 2021 and 2022. Colours indicate a specific crop: dark green indicates the perennial intermediate wheatgrass (IWG) sole crop under organic management, light green indicates intercropped IWG with alfalfa under organic management, orange indicates the biennial ley crop under organic management, light blue indicates annual rye under organic management, dark purple indicates annual wheat under organic management, and light purple indicates annual wheat under conventional (CON) management.

July 2021 and a higher G^- bacteria:G^+ bacteria ratio at 15–30 cm in July 2022 and at 30–40 cm in September 2022 (Fig. 4).

Overall, IWG sole crop had the highest estimated biomass and ratios of microbial groups, conventional annual wheat had the lowest amount, and biennial ley had the medium amount in most combinations of soil depths and sampling time points. The differences among these three cropping systems reflected the trend of microbial biomass and ratios response to the gradients of crop perenniality. The microbial biomass and ratios of IWG-alfalfa intercropping were usually in the middle of that of IWG sole crop and biennial ley, and there was no significant difference ($p > 0.05$) among them in most circumstance. Under few conditions, such as at 5–40 cm in September 2022, IWG-alfalfa intercropping had higher G^- bacteria:G^+ bacteria ratio than IWG sole crop. IWG-alfalfa intercropping also had higher ($p < 0.05$) bacterial growth than biennial ley at almost all depths in September 2022. The soil microbial biomass and ratios of organic rye or wheat were in the middle of that of biennial ley and conventional annual wheat and the difference among them were not statistically significant ($p > 0.05$) in the most circumstance.

3.3. Correlation between microbial biomass carbon and soil total carbon

The soil total carbon concentration was positively correlated with crop perenniality and significantly contributes to the variation of PLFA patterns (Fig. 2). The soil total carbon concentration was higher ($p < 0.05$) in perennial IWG sole cropping and IWG-alfalfa intercropping than in conventional annual wheat cropping at 0–5 cm, and it was also higher ($p < 0.05$) in the upper soil layers compared to the lower soil layers (Fig. 6). The soil total carbon mass was higher ($p < 0.05$) in IWG-alfalfa intercropping than in conventional wheat cropping at 0–5 cm in July 2021, May and July 2022 (Fig. S3). Soil microbial biomass carbon was also higher ($p < 0.05$) in perennial IWG sole cropping than in conventional annual wheat cropping at 0–15 cm, and it was also higher ($p < 0.05$) in the upper soil layers compared to the lower soil layers (Fig. 6). The soil total carbon concentration was positively correlated with soil total microbial biomass carbon ($R^2 = 0.65$, $p < 0.001$) (Fig. S4).

4. Discussion

4.1. Soil microbial community structures shaped by climate-related variables associated with different seasons and soil depths

Climate-related variables (precipitation, soil water content and soil temperature) were found to be important factors affecting the temporal variations in soil microbial community composition and structure. The total amounts of fungi and AM fungi were more abundant in the microbial community under relatively dry and cold soil conditions compared to those under wet and warm conditions (illustrated in Fig. 2), which is in agreement with a comparative study of soil microbial communities under eight land-use types at a larger regional scale (Drenovsky et al., 2010). In this larger scale study, the soil water availability of different land use types was an important factor in structuring soil microbial communities; G^+, sulphate-reducing, anaerobic and general bacterial fatty acids were more abundant in wetter soils, while fungal and G^- bacterial fatty acids were more abundant in drier soils (Drenovsky et al., 2010). A similar pattern of results was obtained in a phylum level study by Castro et al. (2010), who reported that changes in precipitation had a significant impact on bacterial and fungal abundance; specifically, the relative abundance of Proteobacteria was greater in the wet treatments relative to the dry treatments, and Acidobacteria abundance was greater in the dry treatments. Fungi were usually more abundant under dry soil conditions because fungi have a number of traits that can mitigate drought stress and help fungi survive and even grow under dry conditions (Treseder et al., 2010). For example, fungal chitinous cell walls protect fungal cell against the osmotic pressure inside the cell thus maintaining cellular shape (Cortés et al., 2019). Fungal hyphae can be produced slowly but consistently during a dry season enabling fungi to access water and nutrients (Treseder et al., 2010). Fungal mycelia networks can redistribute water along gradients in soil water potential (Guhr et al., 2015). The G^+ bacteria is more abundant under dry soil conditions than G^-, because G^+ bacteria have a thick and interlinked peptidoglycan cell wall which can act as protection towards water stress, while G^- bacteria have a single-layer cell wall and an outer membrane, making them generally more susceptible to water stress (Schimel et al., 2007). Some G^+ bacteria have the ability to form spores, which have the capability to take on a dormant cellular form and thus endure extreme conditions (*e.g.* drought) of their habitat (Andryukov et al., 2020).

The greater total fungal abundance (estimated by PLFAs abundance) found in upper soil layers in our study aligns with the results reported by Mckenna et al. (2020), signifying that the overall richness of fungal operational taxonomic units (OTU) and pathotroph OTU was higher at 0–10 cm than 10–30 cm for IWG sole cropping, annual cropping and native prairie. In our study, the upper soil layer was more enriched in fungi and G^- bacteria while the deeper soil layer was more enriched in general bacteria and G^+ bacteria. This is likely because fungi and G^- bacteria are responsible for decomposing the fresh plant-derived carbon accumulated in the upper soil layer, whereas G^+ bacteria are more capable of using recalcitrant compounds (Kramer and Gleixner, 2006; Tavi et al., 2013), and can assimilate carbon from dead fungal or root biomass that isn't directly from rhizodeposits (Tavi et al., 2013) at deeper soil layers. The saprotrophic fungi were more abundant in the upper soil layer for litter decomposition due to their specific enzymatic activities and high density of hyphae, as reported in other studies (Crowther et al., 2012; Guhr et al., 2015). We see a saprotrophic-to-mycorrhizal shift in fungal composition with increasing soil depth across different cropping systems and sampling times, which is in accordance with the general theory that the litter layer is generally dominated by saprotrophic fungi, while older and deeper layers are increasingly dominated by mycorrhizal fungi (Lindahl et al., 2007; Kyaschenko et al., 2017; Carteron et al., 2021). In this study, these changes in the microbial community's composition were largely driven by differences in soil moisture, water content and soil total carbon concentration, which were higher in the upper soil layers and lower in the deeper soil layers. Nevertheless, half of these variations in soil microbial community still remains unexplained by our model when all significant and measured environmental variables haven been considered. We speculate that part of the unexplained variations may attribute to the unknown environmental variables that were not measured in our study, such as soil inorganic nitrogen, available phosphorus, C:N:P stoichiometry, soil respiration, nutrient mineralization rates, fungal growth rates, root dynamics etc. Nutrient availability, microbial respiration and nutrient mineralization rates could give higher resolution in soil carbon and nitrogen dynamics which can be well correlated with soil microbial community composition shift and microbial activity.

We see a general decrease in the total fungal, total bacterial, AM fungal, and saprotrophic fungal biomass as soil depth increases, and this trend was more pronounced in the perennial cropping systems than in the annual cropping systems. This is likely because the nutrient stratification (*i.e.* variations with soil depth) becomes even more pronounced in perennial cropping due to the lack of tillage compared to annual cropping systems that are tilled frequently. Soil nutrient stratification provides a nutrient-rich environment that supports increased microbial biomass near the soil surface (Helgason et al., 2009). Greater soil nutrient stratification has been found in no-tillage systems due to the accumulation of crop residues and nutrients remaining immobile on the soil surface (Lupwayi et al., 2006). In annual cropping systems, however, the soil bacterial communities can be homogenized by tillage dispersal (West et al., 2023).

4.2. AM fungi-abundant microbial community with higher microbial biomass and activity under perennial cropping systems

The level of crop perenniality plays an important role in shaping the microbial community structure in agricultural soil. According to this study, the higher fungi:bacteria ratio in the perennial cropping systems compared to conventional annual wheat cropping systems in lower soil layers aligns with the results reported by Taylor et al. (2023), which showed that IWG had a higher fungi:bacteria ratio than tilled annual wheat at lower soil layers (15–60 cm). The higher total fungal and AM fungal biomass that our study found in perennial cropping systems compared to annual wheat cropping systems is also in line with the results reported by Duchene et al. (2020), which showed that overall fungi and AM fungi abundance increased in IWG cropping in the topsoil (0–10 cm) compared to annual cropping. The higher fungi proportion and abundance that appeared in cropping systems with high perenniality in our study can be explained by a combination of factors; for example, perennial crops provided long-term (5 yr) soil cover and root exudate input in the absence of tillage practices and low nitrogen fertilizer input.

Our results agree with earlier findings in which long-term land cover together with no-tillage practices had been found to increase fungal biomass (Helgason et al., 2009), fungal diversity (Schmidt et al., 2019) and the fungi:bacteria ratio (Sun et al., 2016) due to reduced physical disturbance and disruption of fungal hyphal network development. Soils with low nitrogen availability generally have fungal-based microbial communities and energy channels (Wardle et al., 2004; de Vries and Bardgett, 2012), whereas nitrogen-rich systems have bacterial-based microbial communities and energy channels, which follows the general assumption that nitrogen demand is predicted by the biomass C:N ratio (~5–15 in fungi compared to ~3–6 in bacteria) (Strickland and Rousk, 2010; Koranda et al., 2014).

The root quality and quantity of the perennial grain crop have been shown to shape soil food webs, and the C:N ratio of IWG coarse root is nearly twice that of annual wheat (Sprunger et al., 2019), suggesting more recalcitrant root tissue input in the soil (Duchene et al., 2020). This facilitates the colonization of fungi more than bacteria because fungi are capable of decomposing more recalcitrant substrates with higher C:N ratios (Hunt et al., 1987; de Vries et al., 2011). Perennial crops host a higher proportion of fungi relative to bacteria compared to annual crops, thus indicating that perennial and biennial cropping have fungal-based food webs while annual cropping has bacterial-based food webs. Except for the drought-resistant potential, which we discussed above in 4.1, fungal-based food webs are generally assumed to benefit other microbial communities since fungi provide assimilable (low molecular weight) substrates and nutrients to the whole microbial community (Beare et al., 1992).

According to this study, the average estimated AM fungi biomass (NLFA 16:1ω5) in perennial IWG sole cropping, which was 6.5 times that of annual wheat cropping, was comparable to the 5-times-greater AM fungal abundance reported by Duchene et al. (2020) in two-year-old IWG cropping compared to the annual rye cropping in France. The higher AM fungi biomass found in our study is attributable to the lack of disturbance of mycorrhizal fungi hyphae by tillage in the perennial cropping systems coupled with the sustained year-round root persistence that continually supplies the carbon resources. Additionally, the low organic nitrogen input in the perennial cropping system may induce and enhance the symbiotic relationship between AM fungi and IWG roots, thereby facilitating plant nutrient acquisition and water uptake. We observed a greater dominance of AM fungi over saprotrophic fungi in perennial IWG sole cropping compared to conventional annual wheat cropping. Furthermore, the AM fungi's dominance was more striking at the deeper soil depths in July and September 2022, likely because the relatively dry soil conditions in September 2022 increased the IWG's reliance on AM fungi for accessing water and nutrients, as shown by Oliveira et al. (2022), who stated that water stress increases the mycorrhizal colonization of a drought-sensitive soybean cultivar in a greenhouse after 3 and 7 days of inoculation. Many studies have also provided evidence that AM fungi alleviate drought and nutrient stress on plant growth (Begum et al., 2019; Gao et al., 2022; Marro et al., 2022). On the other hand, annual wheat crops had less dominant AM fungi, probably due to their very intense management compared to perennial crops, particularly in terms of the higher level of inorganic nitrogen fertilizer used, the frequency of tillage, and the use of fungicides. Moreover, annual wheat shoot was harvested in this study approximately two weeks before the sampling in September. We assume that the plant and mycorrhizal symbiosis in the annual wheat crops was weaker in September due to the lack of any photosynthesis carbon input from plant shoots.

The G^- bacteria:G^+ bacteria ratio, which indicates the relative carbon availability for soil bacterial communities in organic soils (Fanin et al., 2019), was also influenced by the cropping systems. The proportion of G^- bacteria relative to G^+ bacteria in perennial IWG-alfalfa intercropping was higher than that of IWG sole cropping in September 2022, indicating that higher amounts of labile carbon exist in the intercropping system that contains legume alfalfa. This is likely because alfalfa provided labile carbon from root exudates, which favours the G^- bacteria since they are more dependent on simple carbon compounds that are relatively labile and derived from plants, while G^+ bacteria are more dependent on complex carbon compounds derived from soil organic matter that are more recalcitrant (Kramer and Gleixner, 2008; Fanin et al., 2019). The biennial ley crop containing alfalfa and other legumes like white clover and red clover also had a higher G^- bacteria: G^+ bacteria ratio than the IWG sole crop at 15–40 cm in September 2022, likely because the alfalfa together with other legumes in ley cropping increased the rhizosphere volume. The rhizosphere usually harbours more G^- bacteria such as nitrogen-fixing rhizobia and fewer G^+ bacteria (Liang et al., 2011). Furthermore, the increased plant diversity in a plant mixture increases soil moisture, thus promoting the relative abundance of G^- bacteria (Chen et al., 2019).

According to this study, perennial cropping systems significantly increased the total microbial biomass mainly in the upper soil layers (0–30 cm) compared to conventional annual wheat cropping systems, which was contrary to the results reported by Taylor et al. (2024), who found that in deeper (30–60 cm) soil layers, IWG had a higher total microbial biomass than annual wheat. They also found that in the upper (0–15 cm and 15–30 cm) soil layers, IWG had a similar total microbial biomass compared to annual wheat. The higher total microbial biomass in IWG occurring in the upper soil layers in our study was probably due to the distribution of the IWG's roots. As observed during soil sampling for our study and in line with an earlier study by Audu et al. (2022), we found that the IWG roots in our field were more densely distributed in the shallow soil layer (0–5 cm) than in the deeper soil layer (30–40 cm). This also in agreement with a recent study published by Rakkar et al. (2023) showing that an IWG sole crop and IWG-alfalfa intercrops have their highest root biomass in the 0–15 cm soil layer, and the root biomass of the IWG sole crop decreased dramatically in the 15–30 cm, 30–40 cm and 45–60 cm soil layers at the Lamberton and Rosemount research centres in the US in 2018 and 2019. The local soil texture, fertility, pH and climate play important roles in determining IWG root biomass distribution and the biomass of microbial groups. We believe that IWG roots were more densely distributed in the upper soil layers in our research field due to the specific sandy loam soil type and the humid local climate, both of which contributed to the higher total microbial biomass in the upper soil layers. However, the fact that a higher bacterial growth rate was observed at almost all soil depths in the perennial IWG sole cropping compared to annual wheat cropping indicates that bacteria were more active and had a faster biomass turnover at all soil depths in the perennial cropping, likely due to the increased root exudates and soil organic matter input during the IWG growing seasons. The faster turnover also increased the total bacterial biomass and likely the necromass as well, which translated into the observed increase in soil total carbon concentration.

4.3. *Higher crop perenniality reveals greater total carbon accumulation*

For a given carbon concentration and soil thickness, the quantity of soil carbon per unit area depends on soil bulk density, which also varies with management, soil depth and other properties. In our study, if the soil bulk density were higher in perennial IWG cropping than annual cropping due to compaction from lack of tillage, then perennial crops would have a denser soil mass sampled than the annual crop at the same soil depth. Even with a potentially denser soil mass in the perennial crops, the carbon concentration was higher than that in the annual crop plots (where lower bulk density can be assumed), indicating that soil carbon in perennial cropping would been even higher if soil samples were taken and compared at an equivalent soil mass, and our current soil carbon estimates might underestimate the amount of carbon present in the perennial cropping system. Although our carbon estimates might not be ideal, our main finding that perennial crops hold more carbon would not be changed if soil bulk density measurement were applied in the field.

Perennial crops and organic wheat in this study have been applied with organic fertilizer every year. The higher soil carbon in perennial cropping could be partly from the organic fertilizers. However we found that the yearly carbon input from organic fertilizers was only 1.05%–2.81% of soil total carbon at 0–5 cm in perennial cropping, depending on fertilizers' type and amount. The carbon input from organic fertilizer for annual wheat in 2022 was 1.24 %. This 1.24% extra carbon input from organic fertilizer did not significantly influence the soil total carbon accumulation of annual wheat, if we compare the soil carbon concentration and mass between organic annual wheat with conventional annual (that applied only with inorganic fertilizers). In perennial cropping systems, if all carbon from organic fertilizers remained in the soil from 2017 to 2022 and was not respired away by soil microbes, the total carbon input from organic fertilizers to soil total carbon at 0–5 cm would be around maximum 10.6%. Therefore, the higher soil carbon accumulation in perennial cropping than conventional annual wheat were more likely a result of perennial crop itself and management such as no tillage rather than organic fertilizer application. We see a positive correlation between crop perenniality and soil total carbon concentration. Perennial crops continuously provide organic substrates via root exudates and residues for microbes the whole year round, which provides energy for soil microbes and carbon for biosynthesis (Sun et al., 2016), while annual crops provide substrates for four to five months per year. We believe that a larger amount of crop residues and root exudates in perennial cropping systems increases soil microbial biomass and microbial biomass carbon, and thus soil total carbon accumulation. The soil microbial biomass carbon positively correlated with soil total carbon concentration, and perennial cropping systems have higher microbial biomass than annual cropping systems at 0–30 cm, indicating that perennial crops have the potential to increase soil carbon sequestration in shallow soil layers. The greater soil carbon accumulation in perennial cropping systems determined in this study is partly attributable to the fungal-based food webs which have been shown to retain greater ecosystem nitrogen and carbon due to the slower rates of nitrogen cycling (Wardle et al., 2004; de Vries and Bardgett, 2012) and carbon turnover (Holland and Coleman, 1987; Bailey et al., 2002; Six et al., 2006) than bacteria-based food webs. With IWG, the greater soil carbon concentration in shallow soil layers aligns with the results reported by Sprunger et al. (2019), who found that after 4 years, IWG had significantly larger amounts of labile soil carbon and root biomass relative to annual wheat at 0–10 cm. Furthermore, at the deeper soil layer of 30–60 cm, another study (Audu et al., 2022) showed that IWG increased soil organic carbon and microbial biomass and activities compared to organic and conventional annual crops. Our study confirmed that fungal-abundant agricultural soils under perennial cropping sequestered more carbon than bacteria-abundant soils, indicating that soil management through cultivating perennial crops can increase microbial biomass carbon and thus sequestrate more carbon in agricultural soil, although the stabilization of this soil carbon can be influenced by other abiotic and biotic factors which need to be studied further in the future.

5. Conclusion

We conclude that the integration of perennial cereal crops into agroecosystems traditionally dominated by annual crops will enhance soil quality in terms of increased soil microbial biomass, bacterial activity, microbial biomass carbon, and soil total carbon concentration in the upper soil layers. Such changes are likely to improve the quality and fertility of the soil, in favour of quality and quantity of crop production. In addition, it is likely that subsequent reduced tillage activities will facilitate the downward translocation of the soil carbon into the deeper soil profile. Such translocation could potentially support the desired carbon sink function of agricultural soils, as a climate change mitigation strategy. Although climatic conditions are the main drivers of soil microbial communities, our study reveals that at the management level, the cultivation of crops with high perenniality will shape the soil microbial structure towards a more fungal abundant community with higher microbial activity. However, the characteristics of the different fractions of the increased soil total carbon, including the contribution of perennial crop root exudates, as well as the interaction with the soil microbial community and the soil matrix, need to be further researched to better understand the dynamics between the nutrient supply by decomposition and the carbon sequestration capacity, provided by perennial crops. Such understanding is a prerequisite for determining future implementation strategies and science-based practical advice to farmers.

CRediT authorship contribution statement

Shoujiao Li: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ana Barreiro:** Writing – review & editing, Supervision, Methodology, Data curation, Conceptualization. **Juan Pablo Almeida:** Writing – review & editing, Software, Resources, Data curation. **Thomas Prade:** Writing – review & editing, Supervision. **Linda-Maria Dimitrova Mårtensson:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study has been made possible by the Swedish Infrastructure for Ecosystem Science (SITES), particularly the SITES Lönnstorp Research Station at SLU. SITES receives funding through the Swedish Research Council under Grant no. 2017-00635.

This research was performed within the scope of the collaborative project NAPERDIV, which was funded through the 2019–2020 BiodivERsA joint call for research proposals under the BiodivClim ERA-Net COFUND programme, and the Crafoord Foundation 20160622; it also received financial support from the Department of Biosystems and Technology.

The authors thank Ryan Davidson, Eamon Gallagher, Johan Sollerhed, Bindu Sunilkumar, Maria Grudén, Lina Fransson Engman, Linda Groot Nibbelink, Erik Rasmusson, Ida Lager, Kamil Demski and Johannes Rousk for their technical support in establishing the experiment, collecting soil samples, and helping with the soil parameter analyses, gas chromatography analyses and radioactivity analyses. We thank Pedro Rosero for developing the R code that helped identify PLFA indicators. We also thank the anonymous reviewers for their valuable comments and helpful suggestions, which have improved the quality of the paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2024.109621.

Data availability

I have shared the link to my data at the Attach File step.

Data for "Perennial crops shape the soil microbial community and increase the soil carbon in the upper soil layer" (Original data) (Mendeley Data)

References

Andryukov, B.G., Karpenko, A.A., Lyapun, I.N., 2020. Learning from nature: bacterial spores as a target for current technologies in medicine. Sovremennye Tehnologii v Medicine 12, 105. https://doi.org/10.17691/stm2020.12.3.13.

Audu, V., Rasche, F., Dimitrova Mårtensson, L.-M., Emmerling, C., 2022. Perennial cereal grain cultivation: implication on soil organic matter and related soil microbial parameters. Applied Soil Ecology 174, 104414. https://doi.org/10.1016/j.apsoil.2022.104414.

Bååth, E., 1994. Measurement of protein synthesis by soil bacterial assemblages with the leucine incorporation technique. Biology and Fertility of Soils 17, 147–153. https://doi.org/10.1007/BF00337747.

Bååth, E., Pettersson, M., Söderberg, K.H., 2001. Adaptation of a rapid and economical microcentrifugation method to measure thymidine and leucine incorporation by soil bacteria. Soil Biology and Biochemistry 33, 1571–1574. https://doi.org/10.1016/S0038-0717(01)00073-6.

Bailey, V.L., Smith, J.L., Bolton, H., 2002. Fungal-to-bacterial ratios in soils investigated for enhanced C sequestration. Soil Biology and Biochemistry 34, 997–1007. https://doi.org/10.1016/S0038-0717(02)00033-0.

Bajgain, P., Crain, J.L., Cattani, D.J., Larson, S.R., Altendorf, K.R., Anderson, J.A., Crews, T.E., Hu, Y., Poland, J.A., Turner, M.K., Westerbergh, A., DeHaan, L.R., 2022. Breeding intermediate wheatgrass for grain production. In: Plant Breeding Reviews. Wiley, pp. 119–217. https://doi.org/10.1002/9781119874157.ch3.

Barreiro, A., Fontúrbel, M.T., Lombao, A., Martín, A., Vega, J.A., Fernández, C., Carballas, T., Díaz-Raviña, M., 2015. Using phospholipid fatty acid and community level physiological profiling techniques to characterize soil microbial communities following an experimental fire and different stabilization treatments. Catena 135, 419–429. https://doi.org/10.1016/j.catena.2014.07.011.

Barreiro, A., Fox, A., Jongen, M., Melo, J., Musyoki, M., Vieira, A., Zimmermann, J., Carlsson, G., Cruz, C., Lüscher, A., Rasche, F., Silva, L., Widmer, F., Dimitrova Mårtensson, L.M., 2022. Soil bacteria respond to regional edapho-climatic conditions while soil fungi respond to management intensity in grasslands along a European transect. Applied Soil Ecology 170, 104264. https://doi.org/10.1016/j.apsoil.2021.104264.

Barrios, E., 2007. Soil biota, ecosystem services and land productivity. Ecological Economics 64, 269–285. https://doi.org/10.1016/j.ecolecon.2007.03.004.

Beare, M.H., Parmelee, R.W., Hendrix, P.F., Cheng, W., Coleman, D.C., Crossley, D.A., 1992. Microbial and faunal interactions and effects on litter nitrogen and decomposition in agroecosystems. Ecological Monographs 62, 569–591. https://doi.org/10.2307/2937317.

Begum, N., Qin, C., Ahanger, M.A., Raza, S., Khan, M.I., Ashraf, M., Ahmed, N., Zhang, L., 2019. Role of arbuscular mycorrhizal fungi in plant growth regulation: implications in abiotic stress tolerance. Frontiers in Plant Science 10. https://doi.org/10.3389/fpls.2019.01068.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37, 911–917. https://doi.org/10.1139/o59-099.

Brockett, B.F.T., Prescott, C.E., Grayston, S.J., 2012. Soil moisture is the major factor influencing microbial community structure and enzyme activities across seven biogeoclimatic zones in western Canada. Soil Biology and Biochemistry 44, 9–20. https://doi.org/10.1016/j.soilbio.2011.09.003.

Cárceles Rodríguez, B., Durán-Zuazo, V.H., Soriano Rodríguez, M., García-Tejero, I.F., Gálvez Ruiz, B., Cuadros Tavira, S., 2022. Conservation agriculture as a sustainable system for soil health: a review. Soil Systems 6, 87. https://doi.org/10.3390/soilsystems6040087.

Carteron, A., Beigas, M., Joly, S., Turner, B.L., Laliberté, E., 2021. Temperate forests dominated by arbuscular or ectomycorrhizal fungi are characterized by strong shifts from saprotrophic to mycorrhizal fungi with increasing soil depth. Microbial Ecology 82, 377–390. https://doi.org/10.1007/s00248-020-01540-7.

Castro, H.F., Classen, A.T., Austin, E.E., Norby, R.J., Schadt, C.W., 2010. Soil microbial community responses to multiple experimental climate change drivers. Applied and Environmental Microbiology 76, 999–1007. https://doi.org/10.1128/AEM.02874-09.

Chapman, E.A., Thomsen, H.C., Tulloch, S., Correia, P.M.P., Luo, G., Najafi, J., DeHaan, L.R., Crews, T.E., Olsson, L., Lundquist, P.-O., Westerbergh, A., Pedas, P.R., Knudsen, S., Palmgren, M., 2022. Perennials as future grain crops: opportunities and challenges. Frontiers in Plant Science 13. https://doi.org/10.3389/fpls.2022.898769.

Chen, C., Chen, H.Y.H., Chen, X., Huang, Z., 2019. Meta-analysis shows positive effects of plant diversity on microbial biomass and respiration. Nature Communications 10, 1332. https://doi.org/10.1038/s41467-019-09258-y.

Clément, C., Sleiderink, J., Svane, S.F., Smith, A.G., Diamantopoulos, E., Desbrøll, D.B., Thorup-Kristensen, K., 2022. Comparing the deep root growth and water uptake of intermediate wheatgrass (Kernza®) to alfalfa. Plant and Soil 472, 369–390. https://doi.org/10.1007/s11104-021-05248-6.

Contosta, A.R., Frey, S.D., Cooper, A.B., 2015. Soil microbial communities vary as much over time as with chronic warming and nitrogen additions. Soil Biology and Biochemistry 88, 19–24. https://doi.org/10.1016/j.soilbio.2015.04.013.

Cortés, J.C.G., Curto, M.-Á., Carvalho, V.S.D., Pérez, P., Ribas, J.C., 2019. The fungal cell wall as a target for the development of new antifungal therapies. Biotechnology Advances 37, 107352. https://doi.org/10.1016/j.biotechadv.2019.02.008.

Cosentino, S.L., Copani, V., Scalici, G., Scordia, D., Testa, G., 2015. Soil erosion mitigation by perennial species under mediterranean environment. BioEnergy Research 8, 1538–1547. https://doi.org/10.1007/s12155-015-9690-2.

Crowther, T.W., Boddy, L., Hefin Jones, T., 2012. Functional and ecological consequences of saprotrophic fungus–grazer interactions. The ISME Journal 6, 1992–2001. https://doi.org/10.1038/ismej.2012.53.

Culman, S.W., Snapp, S.S., Ollenburger, M., Basso, B., DeHaan, L.R., 2013. Soil and water quality rapidly responds to the perennial grain Kernza wheatgrass. Agronomy Journal 105, 735–744. https://doi.org/10.2134/agronj2012.0273.

Culman, S., Pinto, P., Pugliese, J., Crews, T., DeHaan, L., Jungers, J., Larsen, J., Ryan, M., Schipanski, M., Sulc, M., Wayman, S., Wiedenhoeft, M., Stoltenberg, D., Picasso, V., 2023. Forage harvest management impacts "Kernza" intermediate wheatgrass productivity across North America. Agronomy Journal 115, 2424–2438. https://doi.org/10.1002/agj2.21402.

Dadrasi, A., Chaichi, M., Nehbandani, A., Soltani, E., Nemati, A., Salmani, F., Heydari, M., Yousefi, A.R., 2023. Global insight into understanding wheat yield and production through Agro-Ecological Zoning. Scientific Reports 13, 15898. https://doi.org/10.1038/s41598-023-43191-x.

Daly, E.J., Kim, K., Hernandez-Ramirez, G., Klimchuk, K., 2023. The response of soil physical quality parameters to a perennial grain crop. Agriculture, Ecosystems & Environment 343, 108265. https://doi.org/10.1016/j.agee.2022.108265.

de Oliveira, G., Brunsell, N.A., Sutherlin, C.E., Crews, T.E., DeHaan, L.R., 2018. Energy, water and carbon exchange over a perennial Kernza wheatgrass crop. Agricultural and Forest Meteorology 249, 120–137. https://doi.org/10.1016/j.agrformet.2017.11.022.

de Vries, F.T., Bardgett, R.D., 2012. Plant–microbial linkages and ecosystem nitrogen retention: lessons for sustainable agriculture. Frontiers in Ecology and the Environment 10, 425–432. https://doi.org/10.1890/110162.

de Vries, F.T., van Groenigen, J.W., Hoffland, E., Bloem, J., 2011. Nitrogen losses from two grassland soils with different fungal biomass. Soil Biology and Biochemistry 43, 997–1005. https://doi.org/10.1016/j.soilbio.2011.01.016.

DeHaan, L.R., Ismail, B.P., 2017. Perennial cereals provide ecosystem benefits. Cereal Foods World 62, 278–281. https://doi.org/10.1094/CFW-62-6-0278.

Deng, F., Liang, C., 2022. Revisiting the quantitative contribution of microbial necromass to soil carbon pool: stoichiometric control by microbes and soil. Soil Biology and Biochemistry 165, 108486. https://doi.org/10.1016/j.soilbio.2021.108486.

Dick, C., Cattani, D., Entz, M.H., 2018. Kernza intermediate wheatgrass (Thinopyrum intermedium) grain production as influenced by legume intercropping and residue management. Canadian Journal of Plant Science 98, 1376–1379. https://doi.org/10.1139/cjps-2018-0146.

Drenovsky, R.E., Steenwerth, K.L., Jackson, L.E., Scow, K.M., 2010. Land use and climatic factors structure regional patterns in soil microbial communities. Global Ecology and Biogeography 19, 27–39. https://doi.org/10.1111/j.1466-8238.2009.00486.x.

Duchene, O., Celette, F., Ryan, M.R., DeHaan, L.R., Crews, T.E., David, C., 2019. Integrating multipurpose perennial grains crops in Western European farming systems. Agriculture, Ecosystems & Environment 284, 106591. https://doi.org/10.1016/j.agee.2019.106591.

Duchene, O., Celette, F., Barreiro, A., Dimitrova Mårtensson, L.-M., Freschet, G.T., David, C., 2020. Introducing perennial grain in grain crops rotation: the role of rooting pattern in soil quality management. Agronomy 10, 1254. https://doi.org/10.3390/agronomy10091254.

Ellert, B.H., Janzen, H.H., McConkey, B.G., 2001. Measuring and comparing soil carbon storage. In: LaI, R., Kimble, J.M., Follett, R.F., Stewart, B.A. (Eds.), Assessment Methods for Soil Carbon. Lewis Publishers, Boca Raton London New York Washington. D.C., pp. 131–146

Fanin, N., Kardol, P., Farrell, M., Nilsson, M.-C., Gundale, M.J., Wardle, D.A., 2019. The ratio of Gram-positive to Gram-negative bacterial PLFA markers as an indicator of carbon availability in organic soils. Soil Biology and Biochemistry 128, 111–114. https://doi.org/10.1016/j.soilbio.2018.10.010.

Favre, J.R., Castiblanco, T.M., Combs, D.K., Wattiaux, M.A., Picasso, V.D., 2019. Forage nutritive value and predicted fiber digestibility of Kernza intermediate wheatgrass in monoculture and in mixture with red clover during the first production year. Animal Feed Science and Technology 258, 114298. https://doi.org/10.1016/j.anifeedsci.2019.114298.

Fernandez, C.W., Ehlke, N., Sheaffer, C.C., Jungers, J.M., 2020. Effects of nitrogen fertilization and planting density on intermediate wheatgrass yield. Agronomy Journal 112, 4159–4170. https://doi.org/10.1002/agj2.20351.

Frostegård, A., Bååth, E., 1996. The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. Biology and Fertility of Soils 22, 59–65. https://doi.org/10.1007/BF00384433.

Frostegård, Å., Bååth, E., Tunlio, A., 1993. Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty acid analysis. Soil Biology and Biochemistry 25, 723–730. https://doi.org/10.1016/0038-0717(93)90113-P.

Gao, C., Xu, L., Montoya, L., Madera, M., Hollingsworth, J., Chen, L., Purdom, E., Singan, V., Vogel, J., Hutmacher, R.B., Dahlberg, J.A., Coleman-Derr, D., Lemaux, P.G., Taylor, J.W., 2022. Co-occurrence networks reveal more complexity than community composition in resistance and resilience of microbial communities. Nature Communications 13, 3867. https://doi.org/10.1038/s41467-022-31343-y.

Guhr, A., Borken, W., Spohn, M., Matzner, E., 2015. Redistribution of soil water by a saprotrophic fungus enhances carbon mineralization. Proceedings of the National Academy of Sciences 112, 14647–14651. https://doi.org/10.1073/pnas.1514435112.

Hao, J., Chai, Y.N., Lopes, L.D., Ordóñez, R.A., Wright, E.E., Archontoulis, S., Schachtman, D.P., 2021. The effects of soil depth on the structure of microbial communities in agricultural soils in Iowa (United States). Applied and Environmental Microbiology 87. https://doi.org/10.1128/AEM.02673-20.

Heikkinen, J., Keskinen, R., Regina, K., Honkanen, H., Nuutinen, V., 2021. Estimation of carbon stocks in boreal cropland soils - methodological considerations. European Journal of Soil Science 72, 934–945. https://doi.org/10.1111/ejss.13033.

Helgason, B.L., Walley, F.L., Germida, J.J., 2009. Fungal and bacterial abundance in long-term No-till and intensive-till soils of the northern great plains. Soil Science Society of America Journal 73, 120–127. https://doi.org/10.2136/sssaj2007.0392.

Holland, E.A., Coleman, D.C., 1987. Litter placement effects on microbial and organic matter dynamics in an agroecosystem. Ecology 68, 425–433. https://doi.org/10.2307/1939274.

Hoogsteen, M.J.J., Lantinga, E.A., Bakker, E.J., Groot, J.C.J., Tittonell, P.A., 2015. Estimating soil organic carbon through loss on ignition: effects of ignition conditions and structural water loss. European Journal of Soil Science 66, 320–328. https://doi.org/10.1111/ejss.12224.

Huddell, A., Ernfors, M., Crews, T., Vico, G., Menge, D.N.L., 2023. Nitrate leaching losses and the fate of 15N fertilizer in perennial intermediate wheatgrass and annual wheat — a field study. Science of the Total Environment 857, 159255. https://doi.org/10.1016/j.scitotenv.2022.159255.

Hunt, H.W., Coleman, D.C., Ingham, E.R., Ingham, R.E., Elliott, E.T., Moore, J.C., Rose, S.L., Reid, C.P.P., Morley, C.R., 1987. The detrital food web in a shortgrass prairie. Biology and Fertility of Soils. https://doi.org/10.1007/BF00260580.

Hunter, M.C., Sheaffer, C.C., Culman, S.W., Jungers, J.M., 2020a. Effects of defoliation and row spacing on intermediate wheatgrass I: grain production. Agronomy Journal 112, 1748–1763. https://doi.org/10.1002/agj2.20128.

Hunter, M.C., Sheaffer, C.C., Culman, S.W., Lazarus, W.F., Jungers, J.M., 2020b. Effects of defoliation and row spacing on intermediate wheatgrass II: forage yield and economics. Agronomy Journal 112, 1862–1880. https://doi.org/10.1002/agj2.20124.

Jeffries, P., Gianinazzi, S., Perotto, S., Turnau, K., Barea, J.-M., 2003. The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. Biology and Fertility of Soils 37, 1–16. https://doi.org/10.1007/s00374-002-0546-5.

Joergensen, R.G., Emmerling, C., 2006. Methods for evaluating human impact on soil microorganisms based on their activity, biomass, and diversity in agricultural soils. Journal of Plant Nutrition and Soil Science 169, 295–309. https://doi.org/10.1002/jpln.200521941.

Jungers, J.M., DeHaan, L.R., Betts, K.J., Sheaffer, C.C., Wyse, D.L., 2017. Intermediate wheatgrass grain and forage yield responses to nitrogen fertilization. Agronomy Journal 109, 462–472. https://doi.org/10.2134/agronj2016.07.0438.

Jungers, J.M., DeHaan, L.H., Mulla, D.J., Sheaffer, C.C., Wyse, D.L., 2019. Reduced nitrate leaching in a perennial grain crop compared to maize in the Upper Midwest, USA. Agriculture, Ecosystems & Environment 272, 63–73. https://doi.org/10.1016/j.agee.2018.11.007.

Kaiser, C., Frank, A., Wild, B., Koranda, M., Richter, A., 2010. Negligible contribution from roots to soil-borne phospholipid fatty acid fungal biomarkers 18:2ω6,9 and 18:1ω9. Soil Biology and Biochemistry 42, 1650–1652. https://doi.org/10.1016/j.soilbio.2010.05.019.

Kätterer, T., Andrén, O., Jansson, P.-E., 2006. Pedotransfer functions for estimating plant available water and bulk density in Swedish agricultural soils. Acta Agriculturae Scandinavica, Section B - plant. Soil Science 56, 263–276. https://doi.org/10.1080/09064710500310170.

Kim, K., Daly, E.J., Gorzelak, M., Hernandez-Ramirez, G., 2022. Soil organic matter pools response to perennial grain cropping and nitrogen fertilizer. Soil and Tillage Research 220, 105376. https://doi.org/10.1016/j.still.2022.105376.

Kopittke, P.M., Menzies, N.W., Wang, P., McKenna, B.A., Lombi, E., 2019. Soil and the intensification of agriculture for global food security. Environment International 132, 105078. https://doi.org/10.1016/j.envint.2019.105078.

Koranda, M., Kaiser, C., Fuchslueger, L., Kitzler, B., Sessitsch, A., Zechmeister-Boltenstern, S., Richter, A., 2014. Fungal and bacterial utilization of organic substrates depends on substrate complexity and N availability. FEMS Microbiology Ecology 87, 142–152. https://doi.org/10.1111/1574-6941.12214.

Kramer, C., Gleixner, G., 2006. Variable use of plant- and soil-derived carbon by microorganisms in agricultural soils. Soil Biology and Biochemistry 38, 3267–3278. https://doi.org/10.1016/j.soilbio.2006.04.006.

Kramer, C., Gleixner, G., 2008. Soil organic matter in soil depth profiles: distinct carbon preferences of microbial groups during carbon transformation. Soil Biology and Biochemistry 40, 425–433. https://doi.org/10.1016/j.soilbio.2007.09.016.

Kramer, S., Marhan, S., Haslwimmer, H., Ruess, L., Kandeler, E., 2013. Temporal variation in surface and subsoil abundance and function of the soil microbial community in an arable soil. Soil Biology and Biochemistry 61, 76–85. https://doi.org/10.1016/j.soilbio.2013.02.006.

Kundel, D., Bodenhausen, N., Jørgensen, H.B., Truu, J., Birkhofer, K., Hedlund, K., Mäder, P., Fliessbach, A., 2020. Effects of simulated drought on biological soil quality, microbial diversity and yields under long-term conventional and organic agriculture. FEMS Microbiology Ecology 96. https://doi.org/10.1093/femsec/fiaa205.

Kyaschenko, J., Clemmensen, K.E., Hagenbo, A., Karltun, E., Lindahl, B.D., 2017. Shift in fungal communities and associated enzyme activities along an age gradient of managed Pinus sylvestris stands. The ISME Journal 11, 863–874. https://doi.org/10.1038/ismej.2016.184.

Law, E.P., Wayman, S., Pelzer, C.J., Culman, S.W., Gómez, M.I., DiTommaso, A., Ryan, M.R., 2022a. Multi-criteria assessment of the economic and environmental sustainability characteristics of intermediate wheatgrass grown as a dual-purpose grain and forage crop. Sustainability 14, 3548. https://doi.org/10.3390/su14063548.

Law, E.P., Wayman, S., Pelzer, C.J., DiTommaso, A., Ryan, M.R., 2022b. Intercropping red clover with intermediate wheatgrass suppresses weeds without reducing grain yield. Agronomy Journal 114, 700–716. https://doi.org/10.1002/agj2.20914.

Lehman, R.M., Taheri, W.I., Osborne, S.L., Buyer, J.S., Douds, D.D., 2012. Fall cover cropping can increase arbuscular mycorrhizae in soils supporting intensive agricultural production. Applied Soil Ecology 61, 300–304. https://doi.org/10.1016/j.apsoil.2011.11.008.

Li, S., Barreiro, A., Jensen, E.S., Zhang, Y., Mårtensson, L.-M.D., 2020. Early interspecific dynamics, dry matter production and nitrogen use in Kernza (Thinopyrum intermedium) – alfalfa (Medicago sativa L.) mixed intercropping. Acta Agriculturae Scandinavica Section B Soil and Plant Science 70, 165–175. https://doi.org/10.1080/09064710.2019.1686164.

Liang, C., Sanford, G.R., Jackson, R.D., Balser, T.C., 2011. Potential legacy effects of biofuel cropping systems on soil microbial communities in southern Wisconsin, USA. Agricultural Sciences 2, 131–137. https://doi.org/10.4236/as.2011.22019.

Lindahl, B.D., Ihrmark, K., Boberg, J., Trumbore, S.E., Högberg, P., Stenlid, J., Finlay, R.D., 2007. Spatial separation of litter decomposition and mycorrhizal nitrogen uptake in a boreal forest. New Phytologist 173, 611–620. https://doi.org/10.1111/j.1469-8137.2006.01936.x.

Lupwayi, N.Z., Clayton, G.W., O'Donovan, J.T., Harker, K.N., Turkington, T.K., Soon, Y.K., 2006. Soil nutrient stratification and uptake by wheat after seven years of conventional and zero tillage in the Northern Grain belt of Canada. Canadian Journal of Soil Science 86, 767–778. https://doi.org/10.4141/S06-010.

Ma, W., Tang, S., Dengzeng, Z., Zhang, D., Zhang, T., Ma, X., 2022. Root exudates contribute to belowground ecosystem hotspots: a review. Frontiers in Microbiology 13. https://doi.org/10.3389/fmicb.2022.937940.

Marro, N., Grilli, G., Soteras, F., Caccia, M., Longo, S., Cofré, N., Borda, V., Burni, M., Janoušková, M., Urcelay, C., 2022. The effects of arbuscular mycorrhizal fungal species and taxonomic groups on stressed and unstressed plants: a global meta-analysis. New Phytologist 235, 320–332. https://doi.org/10.1111/nph.18102.

Mårtensson, L.-M.D., Barreiro, A., Li, S., Jensen, E.S., 2022. Agronomic performance, nitrogen acquisition and water-use efficiency of the perennial grain crop Thinopyrum intermedium in a monoculture and intercropped with alfalfa in Scandinavia. Agronomy for Sustainable Development 42, 21. https://doi.org/10.1007/s13593-022-00752-0.

McKenna, T.P., Crews, T.E., Kemp, L., Sikes, B.A., 2020. Community structure of soil fungi in a novel perennial crop monoculture, annual agriculture, and native prairie reconstruction. PLoS One 15, e0228202. https://doi.org/10.1371/journal.pone.0228202.

Moebius-Clune, B.N., Moebius-Clune, D.J., Gugino, B.K., Idowu, O.J., Schindelbeck, R.R., Ristow, A.J., van Es, H.M., Thies, J.E., Shayler, H.A., McBride, M.B., Kurtz, K.S.M., Wolfe, D.W., Abawi, G.S., 2016. Comprehensive Assessment of Soil Health - the Cornell Framework, third ed..2. Cornell University, Geneva, New York.

Mosier, S., Córdova, S.C., Robertson, G.P., 2021. Restoring soil fertility on degraded lands to meet food, fuel, and climate security needs via perennialization. Frontiers in Sustainable Food Systems 5. https://doi.org/10.3389/fsufs.2021.706142.

Ning, Q., Chen, L., Zhang, C., Ma, D., Li, D., Han, X., Cai, Z., Huang, S., Zhang, J., 2021. Saprotrophic fungal communities in arable soils are strongly associated with soil fertility and stoichiometry. Applied Soil Ecology 159, 103843. https://doi.org/10.1016/j.apsoil.2020.103843.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'hara, R., Simpson, G.L., Solymos, P., Stevens, M.H.H., Wagner, H., 2013. Package "vegan". Community ecology package. See chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://cran.r-project.org/web/packages/vegan/vegan.pdf.

Oliveira, T.C., Cabral, J.S.R., Santana, L.R., Tavares, G.G., Santos, L.D.S., Paim, T.P., Müller, C., Silva, F.G., Costa, A.C., Souchie, E.L., Mendes, G.C., 2022. The arbuscular mycorrhizal fungus Rhizophagus clarus improves physiological tolerance to drought stress in soybean plants. Scientific Reports 12, 9044. https://doi.org/10.1038/s41598-022-13059-7.

Olsson, P.A., Bååth, E., Jakobsen, I., 1997. Phosphorus effects on the mycelium and storage structures of an arbuscular mycorrhizal fungus as studied in the soil and roots by analysis of Fatty Acid signatures. Applied and Environmental Microbiology 63, 3531–3538. https://doi.org/10.1128/aem.63.9.3531-3538.1997.

Peixoto, L., Olesen, J.E., Elsgaard, L., Enggrob, K.L., Banfield, C.C., Dippold, M.A., Nicolaisen, M.H., Bak, F., Zang, H., Dresbøll, D.B., Thorup-Kristensen, K., Rasmussen, J., 2022. Deep-rooted perennial crops differ in capacity to stabilize C inputs in deep soil layers. Scientific Reports 12, 5952. https://doi.org/10.1038/s41598-022-09737-1.

Rakkar, M., Jungers, J.M., Sheaffer, C., Bergquist, G., Grossman, J., Li, F., Gutknecht, J.L., 2023. Soil health improvements from using a novel perennial grain during the transition to organic production. Agriculture, Ecosystems & Environment 341, 108164. https://doi.org/10.1016/j.agee.2022.108164.

Ramsey, P.W., Rillig, M.C., Feris, K.P., Holben, W.E., Gannon, J.E., 2006. Choice of methods for soil microbial community analysis: PLFA maximizes power compared to CLPP and PCR-based approaches. Pedobiologia 50, 275–280. https://doi.org/10.1016/j.pedobi.2006.03.003.

Schimel, J., Balser, T.C., Wallenstein, M., 2007. Microbial stress-response physiology and its implications for ecosystem function. Ecology 88, 1386–1394. https://doi.org/10.1890/06-0219.

Schmidt, R., Mitchell, J., Scow, K., 2019. Cover cropping and no-till increase diversity and symbiotroph:saprotroph ratios of soil fungal communities. Soil Biology and Biochemistry 129, 99–109. https://doi.org/10.1016/j.soilbio.2018.11.010.

Six, J., Frey, S.D., Thiet, R.K., Batten, K.M., 2006. Bacterial and fungal contributions to carbon sequestration in agroecosystems. Soil Science Society of America Journal 70, 555–569. https://doi.org/10.2136/sssaj2004.0347.

Söderberg, K.H., Bååth, E., 1998. Bacterial activity along a young barley root measured by the thymidine and leucine incorporation techniques. Soil Biology and Biochemistry 30, 1259–1268. https://doi.org/10.1016/S0038-0717(98)00058-3.

Soumare, A., Diedhiou, A.G., Thuita, M., Hafidi, M., Ouhdouch, Y., Gopalakrishnan, S., Kouisni, L., 2020. Exploiting biological nitrogen fixation: a route towards a sustainable agriculture. Plants 9, 1011. https://doi.org/10.3390/plants9081011.

Sprunger, C.D., Culman, S.W., Robertson, G.P., Snapp, S.S., 2018. How does nitrogen and perenniality influence belowground biomass and nitrogen use efficiency in small grain cereals? Crop Science 58, 2110–2120. https://doi.org/10.2135/cropsci2018.02.0123.

Sprunger, C.D., Culman, S.W., Peralta, A.L., DuPont, S.T., Lennon, J.T., Snapp, S.S., 2019. Perennial grain crop roots and nitrogen management shape soil food webs and soil carbon dynamics. Soil Biology and Biochemistry 137, 107573. https://doi.org/10.1016/j.soilbio.2019.107573.

Strickland, M.S., Rousk, J., 2010. Considering fungal:bacterial dominance in soils – methods, controls, and ecosystem implications. Soil Biology and Biochemistry 42, 1385–1395. https://doi.org/10.1016/j.soilbio.2010.05.007.

Sun, B., Jia, S., Zhang, S., McLaughlin, N.B., Liang, A., Chen, X., Liu, S., Zhang, X., 2016. No tillage combined with crop rotation improves soil microbial community composition and metabolic activity. Environmental Science and Pollution Research 23, 6472–6482. https://doi.org/10.1007/s11356-015-5812-9.

Tavi, N.M., Martikainen, P.J., Lokko, K., Kontro, M., Wild, B., Richter, A., Biasi, C., 2013. Linking microbial community structure and allocation of plant-derived carbon in an organic agricultural soil using 13CO2 pulse-chase labelling combined with 13C-PLFA profiling. Soil Biology and Biochemistry 58, 207–215. https://doi.org/10.1016/j.soilbio.2012.11.013.

Taylor, K., Samaddar, S., Schmidt, R., Lundy, M., Scow, K., 2023. Soil carbon storage and compositional responses of soil microbial communities under perennial grain IWG vs. annual wheat. Soil Biology and Biochemistry 184, 109111. https://doi.org/10.1016/j.soilbio.2023.109111.

Taylor, K.M., Nelsen, T.S., Scow, K.M., Lundy, M.E., 2024. No-till annual wheat increases plant productivity, soil microbial biomass, and soil carbon stabilization relative to intermediate wheatgrass in a Mediterranean climate. Soil and Tillage Research 235, 105874. https://doi.org/10.1016/j.still.2023.105874.

Treseder, K.K., Schimel, J.P., Garcia, M.O., Whiteside, M.D., 2010. Slow turnover and production of fungal hyphae during a Californian dry season. Soil Biology and Biochemistry 42, 1657–1660. https://doi.org/10.1016/j.soilbio.2010.06.005.

Tsiafouli, M.A., Thébault, E., Sgardelis, S.P., de Ruiter, P.C., van der Putten, W.H., Birkhofer, K., Hemerik, L., de Vries, F.T., Bardgett, R.D., Brady, M.V., Bjornlund, L., Jørgensen, H.B., Christensen, S., Hertefeldt, T.D., Hotes, S., Gera Hol, W.H., Frouz, J., Liiri, M., Mortimer, S.R., Setälä, H., Tzanopoulos, J., Uteseny, K., Pižl, V., Stary, J., Wolters, V., Hedlund, K., 2015. Intensive agriculture reduces soil biodiversity across Europe. Global Change Biology 21, 973–985. https://doi.org/10.1111/gcb.12752.

Vestberg, M., Palojärvi, A., Pitkänen, T., Kaipainen, S., Puolakka, E., Keskitalo, M., 2012. Neutral lipid fatty acid analysis is a sensitive marker for quantitative estimation of arbuscular mycorrhizal fungi in agricultural soil with crops of different mycotrophy. Agricultural and Food Science 21, 12–27. https://doi.org/10.23986/afsci.4996.

Wagoner, P., Schaeffer, J.R., 1990. Perennial grain development: past efforts and potential for the future. Critical Reviews in Plant Sciences 9, 381–408. https://doi.org/10.1080/07352689009382298.

Wardle, D.A., Bardgett, R.D., Klironomos, J.N., Setala, H., van der Putten, W.H., Wall, D.H., 2004. Ecological linkages between aboveground and belowground biota. Science 304, 1629–1633. https://doi.org/10.1126/science.1094875.

West, J.R., Lauer, J.G., Whitman, T., 2023. Tillage homogenizes soil bacterial communities in microaggregate fractions by facilitating dispersal. Soil Biology and Biochemistry 186, 109181. https://doi.org/10.1016/j.soilbio.2023.109181.

Willers, C., Jansen van Rensburg, P.J., Claassens, S., 2015. Phospholipid fatty acid profiling of microbial communities-a review of interpretations and recent applications. Journal of Applied Microbiology 119, 1207–1218. https://doi.org/10.1111/jam.12902.

Yan, Y., Wang, C., Zhang, J., Sun, Y., Xu, X., Zhu, N., Cai, Y., Xu, D., Wang, X., Xin, X., Chen, J., 2022. Response of soil microbial biomass C, N, and P and microbial quotient to agriculture and agricultural abandonment in a meadow steppe of northeast China. Soil and Tillage Research 223, 105475. https://doi.org/10.1016/j.still.2022.105475.

Yang, T., Siddique, K.H.M., Liu, K., 2020. Cropping systems in agriculture and their impact on soil health-A review. Global Ecology and Conservation 23, e01118. https://doi.org/10.1016/j.gecco.2020.e01118.

Zhang, S., Huang, G., Zhang, Y., Lv, X., Wan, K., Liang, J., Feng, Y., Dao, J., Wu, S., Zhang, L., Yang, X., Lian, X., Huang, L., Shao, L., Zhang, J., Qin, S., Tao, D., Crews, T. E., Sacks, E.J., Lyu, J., Wade, L.J., Hu, F., 2023. Sustained productivity and agronomic potential of perennial rice. Nature Sustainability 6, 28–38. https://doi.org/10.1038/s41893-022-00997-3.

Zhang, X., Sallam, A., Gao, L., Kantarski, T., Poland, J., DeHaan, L.R., Wyse, D.L., Anderson, J.A., 2016. Establishment and optimization of genomic selection to accelerate the domestication and improvement of intermediate wheatgrass. The Plant Genome 9. https://doi.org/10.3835/plantgenome2015.07.0059.

Supplementary Material

Perennial crops shape the soil microbial community and increase the soil carbon in the upper soil layer

Shoujiao Li[a], Ana Barreiro[b], Juan Pablo Almeida[a], Thomas Prade[a], Linda-Maria Dimitrova Mårtensson[a*]

[a] Swedish University of Agricultural Sciences, Department of Biosystems and Technology, P.O. Box 190, SE-234 22 Lomma, Sweden

[b] University of Santiago de Compostela, Department of Soil Science and Agricultural Chemistry, 27002 Lugo, Spain

*Corresponding author: Linda-Maria Dimitrova Mårtensson

E-mail address: linda.maria.martensson@slu.se, postal address: Department of Biosystems and Technology Box 190, Swedish University of Agricultural Sciences, SE-234 22 Lomma, Sweden

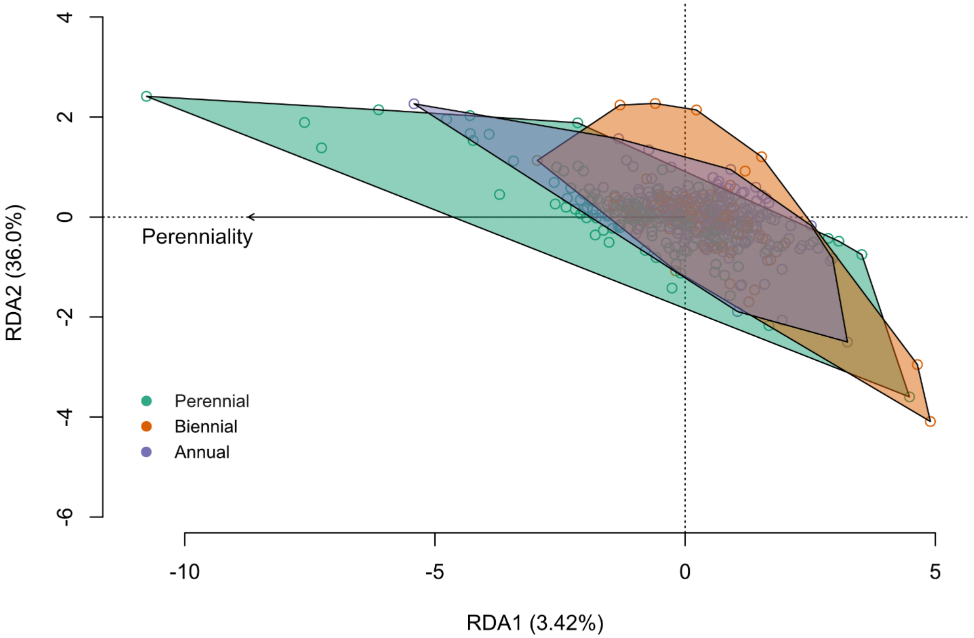


Figure S1. Partial redundancy analysis (pRDA) of soil microbial PLFA profiles in 2021 and 2022, clustered by crop perenniality. Colours indicate crop perenniality: green indicates perennial crops which were five years old, including both the intermediate wheatgrass (IWG) sole crop and the intercrop of IWG with alfalfa, orange indicates biennial crops which were two years old, including the ley crop, and purple indicates annual crops, which were one year old, including the organic winter wheat, rye and conventional winter wheat.

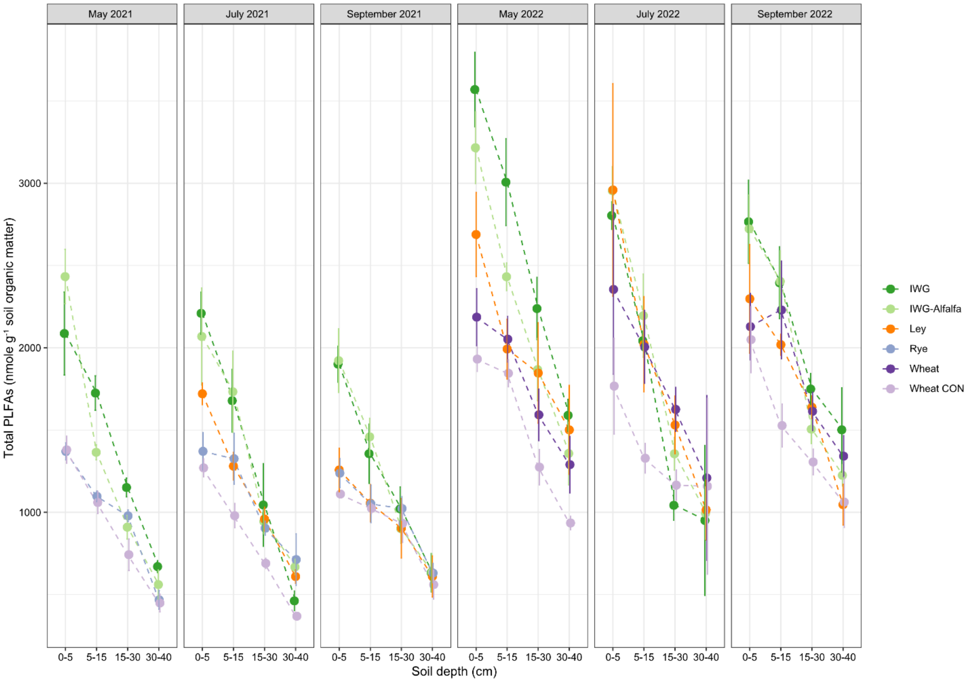


Figure S2. Total microbial biomass (PLFAs) at four soil depths in 2021 and 2022. Dark green indicates perennial Intermediate Wheatgrass (IWG) sole crop under organic management, light green indicates intercropped IWG with alfalfa under organic management, orange indicates biennial ley crop under organic management, light blue indicates annual rye under organic management, dark purple indicates annual wheat under organic management, and light purple indicates annual wheat under conventional (CON) management.

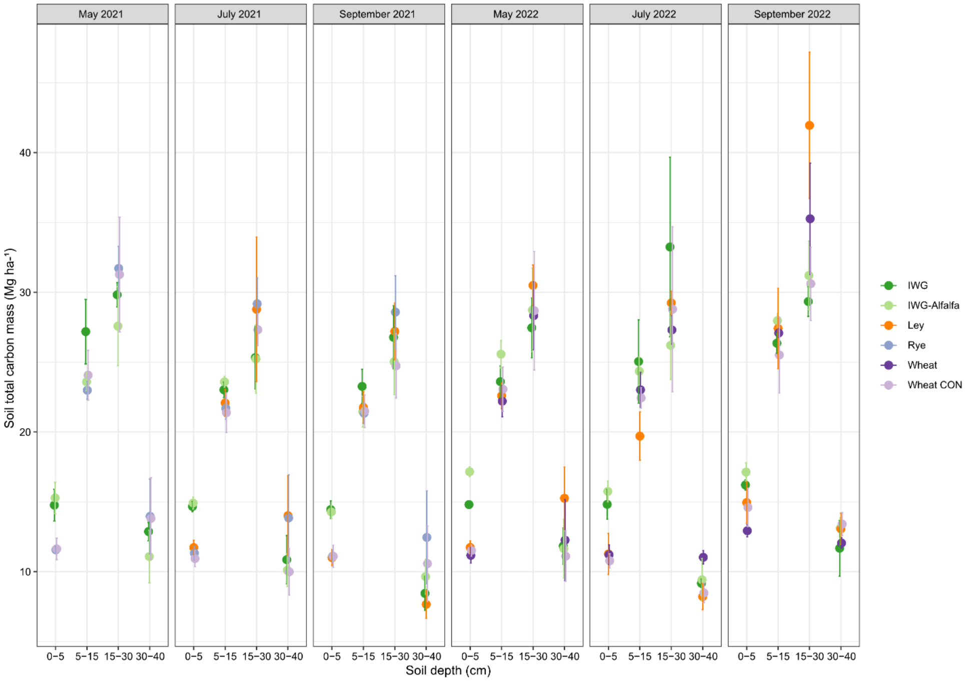


Figure S3. Estimated soil total carbon mass (Mg ha^{-1}) at four fixed soil depth interval in 2021 and 2022. Dark green indicates perennial Intermediate Wheatgrass (IWG) sole crop under organic management, light green indicates intercropped IWG with alfalfa under organic management, orange indicates biennial ley crop under organic management, light blue indicates annual rye under organic management, dark purple indicates annual wheat under organic management, and light purple indicates annual wheat under conventional (CON) management.

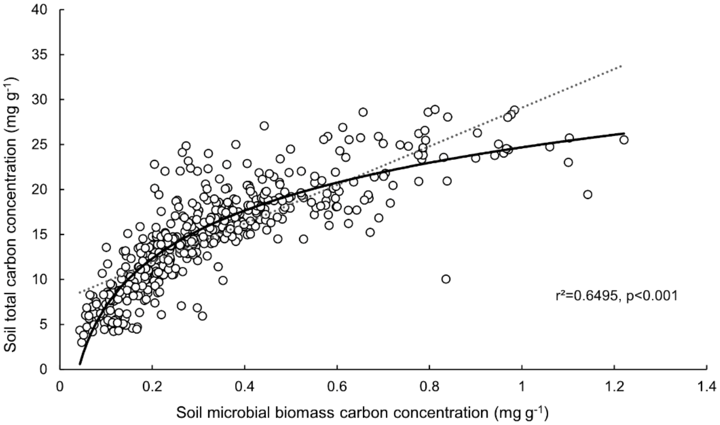


Figure S4. Correlations between the soil microbial biomass carbon and soil total carbon concentration (n = 463).

Table S1. Agronomic management activities of the investigated crops. IWG = intermediate wheatgrass.

Cropping system	Sowing date	ψFertilizer type and level (kg ha^{-1})			Total nitrogen (kg ha^{-1})	*Total carbon (kg ha^{-1})	Pesticide applications
Organic IWG sole crop; IWG-alfalfa intercrop	2 May 2016, complementary sowing in September 2016	2017	Biofer® 9-3-4	444	40	160	no
		2018-2019	biogas digestate	17000	35-40	427	
		2020-2021	Biofer® 10-3-1	500	50	200	
		2022	biogas residue	20000	103	188	
Organic rye	17 Sep 2020	no		0	0	0	no
Organic ley	7 May 2020	no		0	0	0	no
Conventional wheat	17 Sep 2020	Inorganic Yara NPK 20-5-10		850	167	0	herbicides (2.0 L ha^{-1} Boxer and 0.1 L ha^{-1} Diflanil), fungicides (0.7 L ha^{-1} Elatus era)
Organic wheat	25 Sep 2021	biogas residue		20000	103	188	no
Organic ley	14 April 2021	no		0	0	0	0
Conventional wheat	25 Sep 2021	Inorganic Yara NPK 20-5-10		730	141	0	herbicides (2.0 L ha^{-1} Boxer and 0.1 L ha^{-1} Sempra), fungicides (0.7 L ha^{-1} Elatus era)

ψ Organic fertilizers: The Biofer® (from company Gyllebo Gödning) applied to IWG sole crop and IWG-alfalfa intercrop was produced mainly based on animal by-products, manure, slaughterhouse waste, food waste and others. The biogas digestate (from company Gasum Jordberga) applied to IWG and IWG-alfalfa was produced mainly based on plant residues including residues from grain, oil plants, legumes etc. The biogas residue (from company Karpalunds Biogödsel) applied to IWG, IWG-alfalfa and organic wheat was produced based on a mix of manure, slaughterhouse waste, household food waster fat and industrial food waste.

* The total carbon concentration from biogas digestate and residue was estimated based on data from reference Reuland et al., 2022. https://doi.org/10.3390/agronomy12020456

The total carbon conventration from Biofer® was estimated based on product material information; https://www2.jordbruksverket.se/webdav/files/SJV/trycksaker/Pdf_ovrigt/p7_11_2b.pdf

Table S2. Summarized statistics (p-value) of the estimated biomass of microbial organism groups.

	PLFA 18:2ω6	PLFA 16:1ω5	NLFA 16:1ω5	Fungi total	Bacteria Total	G- Bacteria	G+ Bacteria	Actinobacteria
Cropping System	0.006	<0.001	<0.001	0.0007	<0.001	<0.001	0.0033	0.0009
Soil depth	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Sampling time	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Cropping system x soil depth	0.012	0.003	0.8013	0.1923	<0.001	<0.001	0.2410	0.6872
Cropping system x sampling time	0.0004	0.011	<0.001	0.0043	0.00097	0.00043	<0.001	0.1979
Soil depth x sampling time	0.0006	<0.001	<0.001	<0.001	0.00321	0.00035	<0.001	0.0159
Cropping system x soil depth x sampling time	0.393	0.647	0.1900	0.2923	0.8786	0.5479	0.5255	0.4947

Table S3. Summarized statistics (p-value) for bacterial growth, total microbial biomass (total PLFAs), total microbial biomass carbon, soil total carbon concentration and mass, fungi:bacteria ratio, arbuscular mycorrhizal (AM) fungi:saprotrophic fungi ratio, and gram negative (G^-):gram positive (G^+) bacteria ratio.

	Bacterial growth	Total microbial biomass carbon	Total PLFAs	Soil total carbon concentration	Soil total carbon mass	Fungi:bacteria	AM fungi:saprotrophic fungi	G-bacteria:G+ bacteria
Cropping System	0.0017	0.0015	0.0004	0.105	0.4401	0.0042	0.0054	0.0011
Soil depth	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Sampling time	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Cropping system x soil depth	0.5457	<0.001	0.0036	<0.001	<0.001	0.2776	0.2633	0.0101
Cropping system x sampling time	<0.001	0.0007	0.0063	0.0671	0.2213	<0.001	<0.001	<0.001
Soil depth x sampling time	0.0003	0.0137	0.0389	0.3259	0.086	<0.001	0.1707	0.0003
Cropping system x soil depth x sampling time	0.9077	0.9903	0.9604	0.9459	0.9416	0.3977	0.5740	0.2121

Acta Universitatis agriculturae Sueciae
Doctoral Thesis No. 2024:92

Conventional annual monocultures, reliant on synthetic inputs and intensive practices, degrade soil health and are vulnerable to climate change. In contrast, perennial intercropping offers continuous soil cover, deep roots and potential solutions. This thesis explores interactions between perennial cereals, legumes and soil microbes under drought and shows how this partnership stabilises cereal yields, promotes drought-resilient microbial communities, and increases soil carbon storage. Perennial intercropping is emerging as a breakthrough strategy for sustainable agriculture that adapts to and mitigates climate change.

Shoujiao Li received her graduate education at the Department of Biosystems and Technology. She received her undergraduate degree at China Agricultural University.

ISSN 1652-6880
ISBN (print version) 978-91-8046-383-6
ISBN (electronic version) 978-91-8046-419-2