The influence of wheat microbiome on drought stress

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Abstract

Triticum aestivum, the most widely cultivated wheat species, accounts for over 90% of global wheat production, sustaining global food security. However, drought stress is a critical limitation, significantly impacting wheat growth and yield. Arbuscular mycorrhizal fungi (AMF), known for enhancing drought tolerance through mechanisms like improved water and nutrient uptake, have demonstrated potential in alleviating these effects. This thesis explores the impact of AMF inoculation, specifically with *Rhizophagus irregularis* BEG72, on drought resilience and productivity across 30 spring wheat landraces. Wheat landraces, valued for their genetic diversity and local adaptation, are key resources for breeding programmes aimed at enhancing wheat resilience to environmental stresses. This research study involved two irrigation regimes—well-watered (95% field capacity) and drought-stressed (60% field capacity)—and two inoculation treatments: AMF-inoculated and non-inoculated controls. Physiological and morphological parameters, including chlorophyll content, biomass, plant height, and grain yield, were evaluated to determine the contribution of AMF symbiosis to drought resilience.

The findings revealed successful AMF colonisation in all inoculated wheat landraces, significantly enhancing physiological and morphological traits compared to non-inoculated controls. Under well-watered conditions, AMF-inoculated plants exhibited higher chlorophyll content and biomass accumulation, contributing to a marked increase in grain yield. Notably, under drought stress, AMF-inoculated landraces outperformed non-inoculated counterparts, showing significantly greater plant height, biomass, and chlorophyll retention. This translated into an average yield increase of 20% in AMF-inoculated plants compared to controls under drought conditions, accentuating the role of AMF in sustaining productivity when water is limited. These results demonstrate the potential of AMF inoculation as a sustainable approach to improve drought tolerance and yield in wheat. By highlighting the symbiotic benefits of AMF, this research study provides insights valuable for breeding programmes focused on enhancing crop resilience and productivity under increasingly variable environmental conditions linked to climate change.

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In loving memory of my little brother Oliver.

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Author's Declaration

I, Jose Pineda De La O, declare that this thesis is a presentation of original work, and I am the sole author. This work has not previously been presented for an award at this, or any other University. All sources are acknowledged as References.

Chapter 1. General introduction

1.1 Wheat hybridisation

Wheat (*Triticum* spp.) is an indispensable component of diets worldwide, belonging to the Triticeae tribe, which contains approximately 350 species, each with agriculturally significant characteristics. The genus *Triticum* derives its name from the Latin word *tero*, meaning thresh, due to its historical reliance on humans for threshing (Wrigley, 2009; Monneveux et al., 2014; Yilmaz et al., 2022).

Early classifications of wheat date back to Linnaeus in 1753, primarily based on phenological and morphological characteristics. Subsequently, different classification systems emerged, with two of the most influential ones being those developed by Mac Key (1966, 1977) and Dorofeev et al. (1979).

However, the utilisation of these classifications has led to a highly subjective and controversial taxonomy, despite the use of molecular methods to construct the phylogeny. While most western scientists follow the Mac Key classification, eastern scientists tend to adopt the one proposed by Dorofeev (Goncharov, 2011; Dvorak, 2013). Dvorak (2013) further classifies wheat into six biological species, including various domesticated and modern species and subspecies, as summarised in Table 1.1. These classifications, still used today, primarily rely on geographical distribution, although the species and subspecies may occupy different ecological niches (Dvorak, 2013; Spencer & Cross, 2020).

Table 1.1 Summary of the classification of *Triticum* species and subspecies based on their polyploidy levels. Note that two species from the *Aegilops* genus are also included (*) since species of *Triticum* are derived from hybrids with *Aegilops*. Source: (Dvorak, 2013; Lupton, 2014; Monneveux et al., 2014; Molnár-Láng et al., 2015).

PLOIDY LEVEL	GENOME FORMULA	SPECIES	SUBSPECIES	COMMON NAME
DIPLOIDS	AA	*Triticum monococcum*	*aegilopoides*	Wild einkorn wheat
			monococcum	Cultivated einkorn wheat
		Triticum urartu		Wild urartu wheat
	BB	**Aegilops speltoides*		-
	DD	**Aegilops tauschii*		-
TETRAPLOIDS	AABB	*Triticum turgidum*	*dicoccoides*	Wild emmer wheat
			dicoccum (syn. *diccocon*)	Cultivated emmer wheat
			durum	Durum or hard wheat
			turgidum	Pollard wheat
			turanicum	Khorassan wheat
			polonicum	Polish wheat
			carthlicum	Persian wheat
			paleocolchieum	Georgian wheat
			isphahanicum	-
	AAGG	*Triticum timopheevii*	*timopheevii*	Domesticated timopheevii
			armeniaeum (syn. *araraticum*)	Wild timopheevii
HEXAPLOIDS	AABBDD	***Triticum aestivum***	***aestivum***	Bread wheat
			compactum	Club wheat
			macha	-
			spelta	Spelt wheat
			sphaerococcum	Indian dwarf wheat
			tibetanum	Tibetan wheat
			vavilovii	-
			yunanense	Yunan wheat
	AAAAGG	*Triticum zhukovskyi*		-

Traditionally, wheat classification has been based on ploidy levels, which refer to the presence of multiple genomes within a single cell. This phenomenon is widespread in the plant kingdom, and it is considered that all plant species evolved from one or more episodes of polyploidisation. Polyploidisation is associated with the domestication of many crops, as polyploids often exhibit agriculturally favourable characteristics (Glémin & Bataillon, 2009; Dvorak, 2013; Zhang et al., 2019).

Polyploid plants can be categorised as either autopolyploids, where chromosome numbers are duplicated within the same species, or allopolyploids, resulting from the hybridisation of two or more genomes from different species (Zhao et al., 2021).

For over a century, it has been known that wheat is an allopolyploid genus with seven chromosomes, which contain three ploidy levels: diploids (2n = 2x = 14), tetraploids (2n = 4x = 28), and hexaploids (2n = 6x = 42) (Table 1.1). In this notation, 2*n* represents the number of chromosome copies in the somatic cells, *x* signifies the seven copies of the chromosomes, and the last number denotes the total number of chromosomes per cell (Arendt & Zannini, 2013; Tuberosa et al., 2014).

Triticum aestivum, formally known as *T. aestivum* L. subsp. *aestivum* or common bread wheat, is a hexaploid species with a genomic formula of AABBDD. This indicates that it possesses three distinct diploid genomes originated from three sources: *Triticum urartu* (AA) and two closely related species, *Aegilops speltoides* (BB) and *Aegilops tauschii* (DD) (Mayer et al., 2014; Xu et al., 2019).

It has been hypothesised that allopolyploidisation between the A and B genomes occurred first, giving rise to the tetraploid *T. turgidum* (AABB). Subsequently, hybridisation with the D genome led to the formation of modern bread wheat approximately 8000 to 8500 years ago (Figure 1.1) (Mayer et al., 2014; Xu et al., 2019).

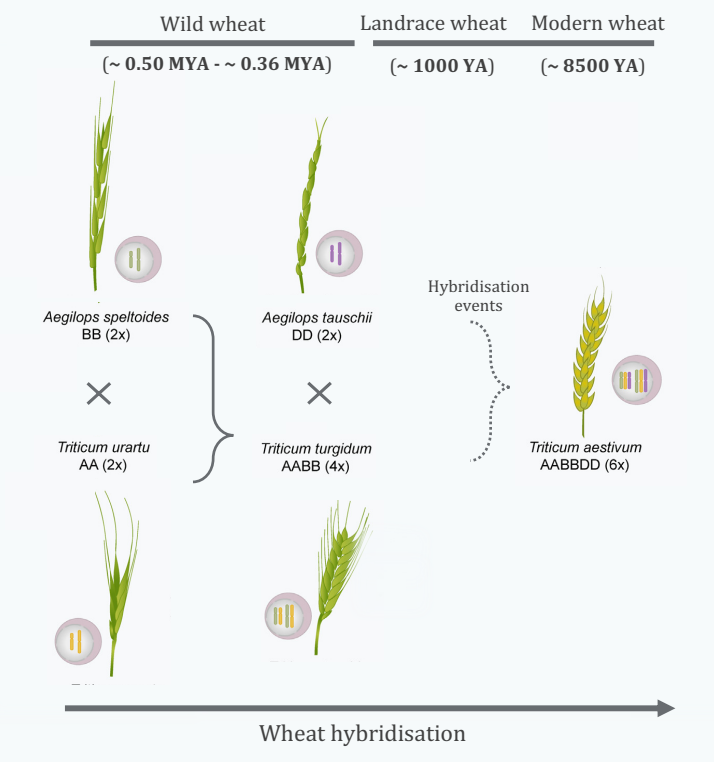


Figure 1.1 | Schematic illustration of the evolution of hexaploid wheat, *Triticum aestivum*, through a series of hybridisation events. Estimated times of species origin are shown in million years ago (MYA) and years ago (YA). Modified from Awan et al., 2022.

1.2 Wheat development

Development in plants refers to the sequence of stages in their life cycle, such as germination, flowering, and maturity. This process is distinct from growth, which comprises the increase in size and biomass. Traditionally, development in wheat is divided into distinct phases, depending on different authors (Feekes, 1941; Haun, 1973). Zadoks et al. (1974), created a scale using a two-digit, computer-compatible decimal format to describe 100 stages of the wheat life cycle, from germination to ripening (Table 1.2). The first digit of the Zadoks scale represents a broad, self-explanatory growth stage, while the second digit provides specific details within that stage, allowing for precise description of the development of the plant. This two-digit system permits detailed description about the phenological status of the (wheat) plants (Zadoks et al., 1974; Hyles et al., 2020).

Table 1.2 Summary of general stages of the Zadoks scale for wheat development. Modified from Zadoks et al. (1974).

CODE	DESCRIPTION	SCALE	STAGE RANGE
0	**Germination**: From dry seed to seedling emergence	00-09	00: Dry seed, 07: Coleoptile emerged, 09: Leaf just at coleoptile tip
1	**Seedling growth**: From first leaf to the full emergence of three leaves	10-19	10: First leaf through coleoptile, 13: Three leaves unfolded
2	**Tillering**: Formation and development of tillers	20-29	21: Main shoot and 1 tiller, 25: Main shoot and 5 tillers
3	**Stem elongation**: Jointing stage, where the stem starts to grow	30-39	31: First node detectable, 39: Flag leaf ligule/collar visible
4	**Booting**: The head of the wheat is enclosed in the flag leaf sheath	40-49	41: Flag leaf sheath extending, 49: First awns visible
5	**Heading**: The head or spike emerges from the sheath	50-59	51: First spikelet of inflorescence visible, 59: Emergence of inflorescence completed
6	**Flowering (Anthesis)**: The period when pollination occurs	60-69	61: Beginning of anthesis, 69: Anthesis completed
7	**Milk development**: Grain filling stage where grains start forming	70-79	71: Early milk, 77: Late milk
8	**Dough development**: Grains gain weight and change texture	80-89	83: Early dough, 87: Hard dough
9	**Ripening**: Final maturation of the grain	90-99	91: Grain hard (difficult to divide by thumbnail), 99: Fully ripe

In this scale, the code 0 (Z00-09) designates germination, including the period from the dry seed until the appearance of the coleoptile. This stage in wheat begins with embryo germination, initiated by breaking seed dormancy, typically triggered by exposure to low temperatures (minimum 4°C) and a moisture stimulus (around 35% to 45% humidity). Germination is completed when a part of the embryo, generally the radicle, elongates to penetrate the surrounding substrate (Bewley, 1997; Gooding, 2009).

During the first stages of germination, approximately three to six primary or seminal roots emerge, followed by the production of nodal or crown roots within one or two months, which constitute 90% to 95% of the total root volume. Seminal and crown roots then proliferate to form lateral roots (Eshel & Beeckman, 2013).

Traditionally, the role of the root system was only associated with anchorage and the uptake of water and mineral nutrients. However, it is now recognised as key to plant growth, development, health, and productivity. Additionally, it can influence soil structure and interactions with other organisms (Grzesiak et al., 2019).

As the root system develops, the coleoptile, a structure that covers the first leaf and shoot stem, expands. The coleoptile protects the leaf primordia during their growth through the soil and ceases once the surface is reached due to the light response (Buntin et al., 2009; Gooding, 2009; Lupton, 2014).

Code 1 designates seedling growth, up to tillering (Z10-19). The seedling develops a leaf, also known as blade or lamina, as in other cereals, possesses a leaf sheath in which the union point, the auricles (1 to 3 mm long and hairy), are differentiated (Figure 1.2). True leaves (not coleoptilar) are designated with a letter and a number following chronological formation (e.g., L1, L2, L3, etc.) until the appearance of the last one, the flag leaf. The number, size, and shape of the leaves depend on various factors such as temperature, photoperiod, nutrient accessibility, and plant variety (McMaster, 2009).

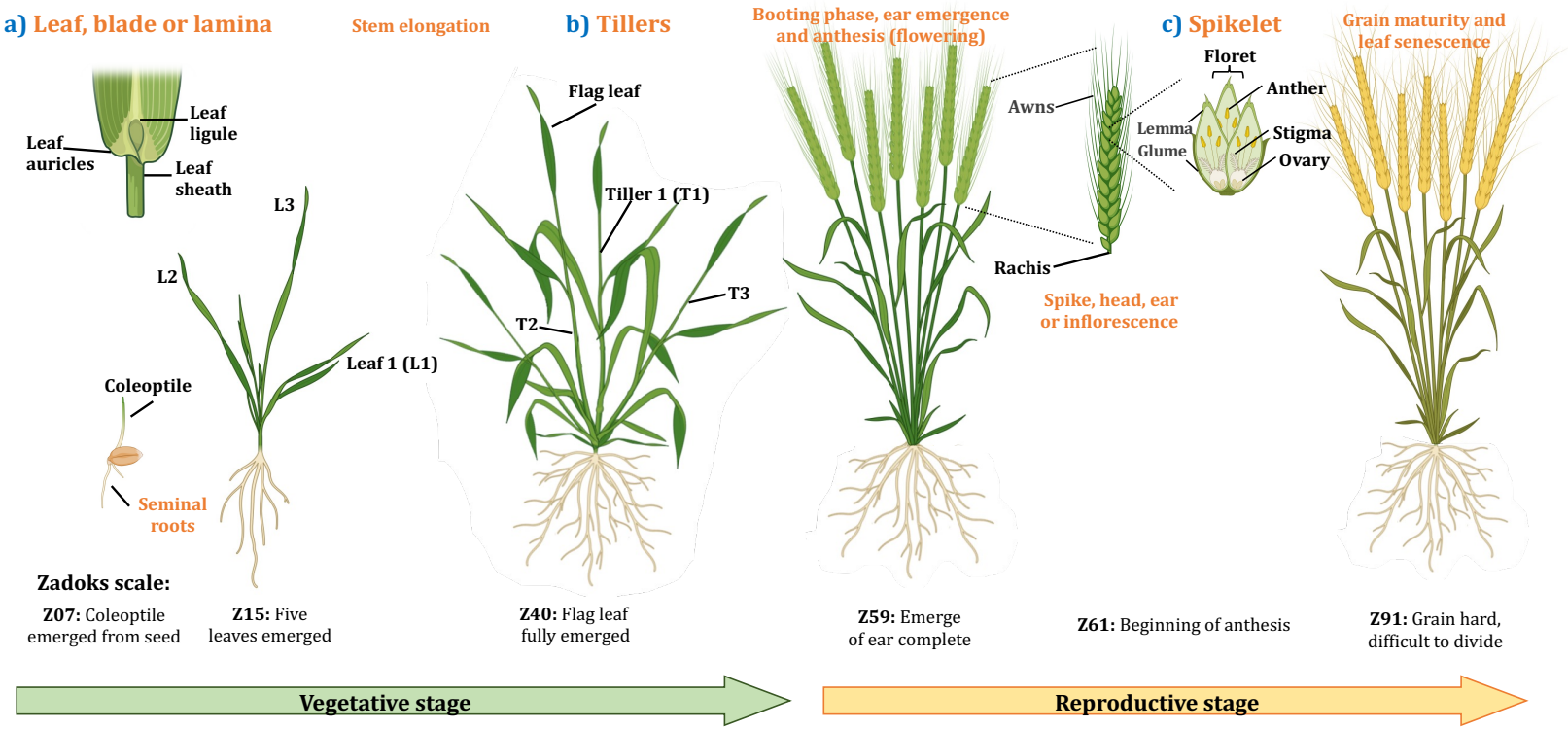


Figure 1.2 | Schematic representation of the vegetative (green arrow) and reproductive (yellow arrow) stages of wheat, and Zadoks scale stages. Embryo germination initiates the emergence of seminal roots and the coleoptile, a structure that covers the first leaf and shoot stem. a) The leaf, blade, or lamina has a leaf sheath in which the auricles serve as union points. b) Tiller buds located in the axils of the leaves develop into new tillers (branches). Leaves and tillers are designated with a letter and a number following chronological formation (e.g., L1, L2, L3 and T1, T2, T3, respectively). c) Wheat transitions to the reproductive stage by forming inflorescences, comprising a group of spikelets consisting of lemma, glume, awn, and reproductive organs. Once fertilised, the grain-filling stage commences until grain maturity, culminating in grain dormancy and leaf senescence. Created with BioRender.com (2023).

Code 2 indicates the beginning of tillering (Z20-29). Tillering refers to the degree of branching that establishes the number of spikes (inflorescences) per unit area, ultimately influencing crop yield (Dreccer et al., 2013). Tiller buds located in the axils of the leaves develop to form new tillers. During their early development, tillers are bound to the leaf sheaths and depend on the parent shoot for nutrients. After the growth of three leaves and a root system, tillers become independent (Buntin et al., 2009; Lupton, 2014).

Tillers are named in the same way as the leaves, with a letter and a number (Figure 1.2b). The primary tiller will receive the same designation as the corresponding leaf number; for example, T1 is the tiller that has emerged from the first leaf (L1), the secondary tiller will be named T2, and subsequent tillers will follow that sequence (Lupton, 2014; Hyles et al., 2020).

This growth pattern allows the plant to expand and form more inflorescences. The tiller number, size, survival, and fertility are related to different environmental factors such as temperature, light intensity, plant density, and nutrient availability (Gooding, 2009; Hyles et al., 2020).

Code 3 indicates stem elongation (Z30-39) including the appearance of the flag leaf. Wheat plants transition to the reproductive stage, entering to the booting process (Z40-49), marked by the development of the inflorescences (Z50-59), which emerge from the sheath of the flag leaf (Kavamura et al., 2021).

The anthesis or flowering stage (Z60-69) is characterised by the spikelets opening, exposing the reproductive organs, typically anthers are more visible. The reproductive organs first develop in the middle of the spike, subsequently, development progresses to the tip and base of the spike (Figure 1.2c) (Kavamura et al., 2021).

Spike pollination begins three to eight days after spike emergence, with spikelets undergoing self-pollination, although cross-pollination is also possible (McMaster, 2009; Shewry et al., 2009). The pollination involves the contact of the pollen grains

developed in the stamen with the receptive stigma, where the pollen adheres, hydrates and germinates. This results in the formation of a pollen tube that carries sperm cells that grow through the stigma to the ovule within the ovary (Lord & Russell, 2002).

After spike pollination, the grain development or grain filling stage commences, code 7 (Z70-80). This process involves the biosynthesis and accumulation of photoassimilates (starch and proteins), and other elements, including vitamins, minerals, and fibres. These reserves are crucial for the future germination and establishment of the grain. Starch accumulation is influenced by environmental factors, with moisture and temperature playing a significant role in determining the final dry weight of the grain (Lv et al., 2021).

Three phases can be described during grain filling. The first phase begins with grain enlargement, involving cellular multiplication, expansion, and the accumulation of water, which is used to transport starch and other nutrients. The flag leaf, serving as the main source, contributes approximately 40% of the photosynthates to the grain. Additionally, soluble reserves derived from senescing vegetative organs also contribute (Buntin et al., 2009; Xie et al., 2015).

One week after anthesis, A-type starch granules (the largest class with a diameter >10 μm) and B-type granules (diameter <10 μm) begin to accumulate in the grain. Subsequently, ten days after the anthesis initiation, proteins in the form of spherical bodies (0.5 to 1.5 μm), derived from the Golgi apparatus, accumulate (Shewry et al., 2009; Zhang et al., 2016).

Simultaneously, 10 to 30 days after anthesis initiates the second phase, characterised by the accumulation of dry biomass. During this stage, numerous protein bodies start to fuse, forming a protein matrix that combines with the starch granules. At this point, the maximum water content in the grain is reached (Xie et al., 2015).

The third phase marks the culmination of grain filling (Z80-89), characterised by the maximum accumulation of dry biomass in each grain and a subsequent loss of water. Finally, code 9 (Z90-99) indicates the physiological maturity of the grain, accompanied by senescence of the flag leaf, which acquires the characteristic straw colour, and the subsequent grain dormancy (Buntin et al., 2009; Gooding, 2009; Xie et al., 2015).

Although the entire lifecycle of wheat is likely affected by environmental stresses, such as drought, the pre-anthesis and grain filling stages exhibit greater sensitivity. Drought stress imposed during the vegetative stage can negatively affect plant development while having little to no effect on grain yield; these effects can be ameliorated or even reversed if plants are rewatered. However, if drought stress occurs during the reproductive stage, grain yield is significantly reduced, even if the plants are rewatered, as this effect becomes irreversible at that point (Farooq et al., 2014; Yu et al., 2019).

1.3 Wheat landraces

Plant landraces emerged alongside agriculture and horticulture approximately 10,000 years ago. Due to the complex nature of landraces, it is not feasible to establish a singular definition that encompasses all agricultural crops, vegetables, ornamental plants, and trees (Zeven, 1998).

The term variety is commonly used by many authors as a synonym for plant landrace. Furthermore, some authors have added the name of a specific region, where landraces have been grown. However, a demarcation between the terms landrace and cultivar has been made. The primary difference is that yield stability is the main characteristic of a landrace under its particular conditions, whereas a cultivar is distinguished by its high yielding capacity under optimal conditions (Zeven, 1998).

Wheat landraces are traditionally defined as local wheat varieties that have experienced both natural and artificial selection over time in specific geographic regions to meet the ethnic, traditional, and environmental needs of the local population (Villa et al., 2005; Jaradat, 2013; Lodhi et al., 2020; Ozturk & Gul, 2020).

Approximately 100 to 150 years ago, prior to the Green Revolution, wheat landraces were the only 'cultivars or varieties' available to farmers. The Green Revolution, which occurred between 1940 and 1960, was characterised by the implementation of new agricultural practices and technologies. Before this period, in 1935, reduced height (*Rht*) genes of a semi-dwarf variety of wheat (Norin 10) were incorporated into wheat plants to provide resistance to lodging. In wheat, two types of lodging have been identified: stem lodging, characterised by bent and/or broken lower internodes, and root lodging, which results in unbroken but leaned internodes due to inadequate anchorage in the soil or by the plant weight. In both cases, adverse weather conditions such as rain, wind, and hailstorm can cause lodging (Gale et al., 1981; Sterling et al., 2003; Khobra et al., 2019; Asami, 2023).

This dwarf wheat variety was later propagated to produce high-yielding, semi-dwarf winter wheat varieties. In 1953, Dr Norman Borlaug made crosses with Mexican wheat landraces to produce a more productive dwarf variety. This new variety was less prone to lodging and allowed for higher applications of nitrogen fertilisers (Asami, 2023).

Dr Borlaug, known as the father of the Green Revolution, was awarded the Nobel Peace Prize in 1970 for his work. The introduction of the new dwarf wheat varieties to regions with similar environmental conditions, agricultural practises, and technologies significantly improved food security in developing countries and increased world wheat productivity five-fold (Gale et al., 1981; Braun et al., 1997; Marcusee et al., 2014; Molnár-Láng et al., 2015; Lopes et al., 2015; Grillo et al., 2017; Asami, 2023; Feldman & Levy, 2023).

Moreover, improved wheat varieties have demonstrated advantages in grain production under abiotic stresses such as drought and extreme temperatures, as

well as in challenging soil conditions like extreme pH levels, salinity, toxicity, and nutrient deficiencies (Feldman & Levy, 2023). However, the reliance on a relatively small number of wheat varieties has had a contrary effect: the loss of well-adapted plants and genetic diversity, leading to phenotypic homogeneity (i.e., plants being at the same growth stage) (Lopes et al., 2015).

It has been hypothesised that the loss of genetic diversity in wheat is related to the relatively few crosses between tetraploid and diploid progenitors of bread wheat. Another hypothesis suggests that the use of a relatively limited number of parent lines in breeding programmes has reduced the population size, thereby limiting germplasm exchange (Lopes et al., 2015).

Phenotypic homogeneity in wheat varieties offers advantages for the mechanisation of various agronomic practices, such as pest and disease management (including the application of herbicides, pesticides, and chemical fertilisers), efficient harvesting, and varietal identification (Grillo et al., 2017). However, this homogeneity also involves potential negative aspects that can affect the entire crop. For instance, when wheat plants are phenotypically similar, they are more likely to be susceptible to specific diseases or environmental stresses, such as drought, due to the lack of genetic diversity (Grillo et al., 2017).

1.3.1. Use and identification of wheat landraces

Contrary to wheat varieties, wheat landraces are considered valuable sources of genetic variability in breeding programmes. They can introduce new genes or alleles into wheat cultivars, leading to the development of new hybrids with improved environmental adaptability, disease resistance, higher yields, and enhanced grain quality. Consequently, the identification and characterisation of wheat landraces using morphological, biochemical, or molecular methods play a crucial role in exploiting these genetic resources (Wingen et al., 2014; Lopes et al., 2015; Reynolds & Langridge, 2016; Gharib et al., 2021).

Morphological characterisation of wheat landraces involves observing and measuring physical features such as plant height, spike length, glume length, and grain size. For instance, Grillo et al. (2017) implemented a morphological approach to study the phenotypic characteristics of glumes, including size, shape, colour and texture, using computerised image analysis techniques. This approach proved valuable for identifying commercial wheat varieties, and wheat landraces as source of genetic diversity (Grillo et al., 2017).

Moreover, it is suggested that a morphological characteristic known as free threshing, or naked grain, is present in wheat landraces. In these landraces, spikes at maturity easily disintegrate from the rachis, with awns (long structures that insert into the ground after falling) facilitating self-cultivation (McMaster, 2009; Shewry et al., 2009). In contrast, (modern) wheat varieties do not exhibit this feature; they remain hulled (enclosed by the palea, lemma, and awn) and more tightly attached to the rachis, which extends the harvesting period and reduces grain losses. However, morphological traits can vary based on environmental conditions and among different wheat landraces (McMaster, 2009; Shewry et al., 2009).

Molecular genetic markers serve as valuable tools for identifying genes in wheat landraces. Simple sequence repeats (SSRs), which are highly polymorphic, are employed to identify and differentiate genotypes within species such as wheat. SSRs offer advantages over other markers due to their simplicity of analysis, high reproducibility, and user-friendliness. They have been extensively used to characterise genetic diversity in seed bank collections of wheat germplasm, landraces, and wild relatives. For example, Dreisigacker et al. (2005) used 44 SSRs to differentiate individual wheat landraces, identifying 256 alleles across 36 accessions from various countries (Dreisigacker et al., 2005).

Overall, wheat landraces are considered a reservoir of valuable traits due to their high adaptability to changing climatic conditions. Their crossability makes them a promising and preferred genetic resource for breeders. Wheat landraces can be used as donors to improve specific traits and as recipients to enhance yield production. However, the latter is only possible with pre-breeding efforts, as wheat

landraces typically produce less but possess a superior genetic background (Adhikari et al., 2022).

1.4 Wheat growth habits

Although both wheat landraces and cultivars are adapted to conventionally managed environments (i.e., typical agricultural practices, which often involve the use of synthetic fertilisers, pesticides, and irrigation), they frequently face challenges posed by varying environmental conditions. The adaptation and development of the crop to new regions is regulated by the timing of flowering, which is determined by three groups of loci: vernalisation (*Vrn*) genes, which are associated with the plant requirement for low temperatures to transition from the vegetative to the reproductive stage; photoperiod (*Ppd*) genes, responsive to day length; and earliness *per se* (*Eps*) genes, operating independently of both vernalisation and photoperiod genes (Shavrukov et al., 2017; Afshari-Behbahanizadeh et al., 2024).

Vernalisation is a physiological process that occurs in certain plants growing in cold regions, typically between 0 to 10°C, followed by a relatively short growing season. This common phenomenon involves plants remaining in the vegetative stage to delay the flowering process, thereby avoiding potential damage caused by cold temperatures (Xu & Chong, 2018).

Moreover, vernalisation requirements are largely controlled by *VRN1*, *VRN2*, *VRN3,* and *VRN4*, which play a crucial role in controlling wheat growth habits. Among these, *VRN1* alleles: *VRN-A1*, *VRN-B1*, and *VRN-D1*, located on chromosomes 5A, 5B, and 5D, respectively are considered essential promoters of flowering (Dragovich et al., 2021; Bloomfield et al., 2023). The presence of at least one dominant allele of *VRN1* results in a spring or in a facultative (also known as semi-winter) growth habit, which are no sensitive or partially sensitive to vernalisation, respectively (Lin et al., 2008; Kamran et al., 2014; Xu & Chong, 2018).

On the other hand, when a plant carries three recessive alleles (*vrn1*, *vrn2*, and *vrn3*), it exhibits a winter growth habit. Winter wheat usually requires a vernalisation period lasting between six to eight weeks, while facultative wheat plants typically need about two to four weeks. Spring wheat may or may not require a short vernalisation period (Meng et al., 2016).

Winter wheat, sown in autumn, experiences low temperatures (the period before vernalisation), but the expression of the *VRN1* gene remains low, preventing the transition from the vegetative to the reproductive stage (Figure 1.3). After wheat germination in autumn, with relatively long days, *VRN2* levels become high, repressing *VRN3*, which consequently impedes the induction of *VRN1*, delaying the flowering process (Pidal et al., 2009; Xu & Chong, 2018; Hyles et al., 2020; Sharma et al., 2020).

During winter (the period of vernalisation), days are shorter and colder, inhibiting flowering. During this period, *VRN1* levels are very low in leaves and apices but gradually upregulated, resulting in the downregulation of *VRN2*. In spring, winter wheat requires a combination of vernalisation and the photoperiod (day length) response, controlled by three homoeologous genes *PPD-A1*, *PPD-B1*, and *PPD-D1*. However, the photoperiodic response in bread wheat is mainly governing by the *Ppd-D1* allele, followed by *Ppd-B1* and *Ppd-A1*, respectively, which accelerates flowering in long days (Chen et al., 2013; Shavrukov et al., 2017; Hyles et al., 2020).

The combination of low levels of *VRN2* and the perception of the day length (long days) by the *PPD-D1* (*PPD1*) gene accelerates the upregulation of *VRN3*, a promoter of flowering. Subsequently, *VRN3* promotes high *VRN1* transcription levels in the shoot apex for the induction of the flowering (Pidal et al., 2009; Molnár-Láng et al., 2015; Xu & Chong, 2018; Hyles et al., 2020; Sharma et al., 2020).

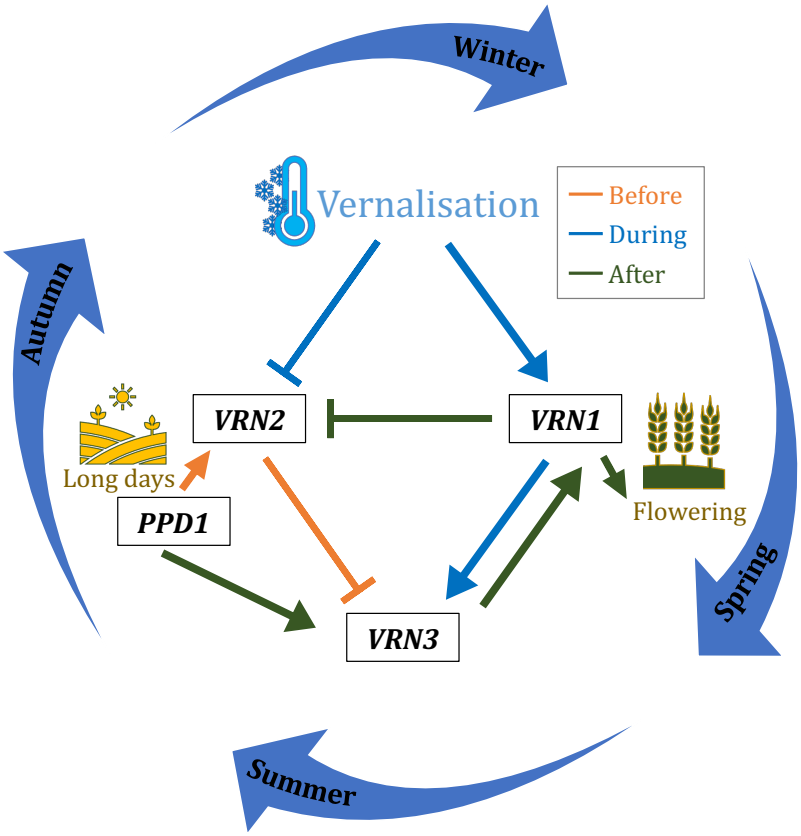


Figure 1.3 | Interactions among vernalisation genes (*VRN1*, *VRN2*, and *VRN3*) in winter wheat. After wheat germination in autumn (during relatively long days), *VRN3* is repressed by high levels of *VRN2*, impeding the induction of *VRN1*. Initially, *VRN1* is transcribed at very low levels in leaves and apices, gradually upregulating during the short, cold days of winter, consequently downregulating *VRN2*. The low levels of *VRN2* transcripts in the leaves facilitate the upregulation of *VRN3* by long days in the spring, a process mediated by photoperiod (*PPD1*) genes. Then, *VRN3* is exported to the shoot apex, where it promotes high *VRN1* transcription levels for the induction of flowering. Arrows indicate induction, and crossed bars indicate repression, with colours indicating the period of vernalisation. Modified from Xiao et al., 2022.

Spring wheat is typically sown in spring to develop and mature just before the onset of autumn snowfall. However, in regions with mild winters, such as South Asia, North Africa, and the Middle East, "spring wheat" grows best when sown in autumn. This confusion arose from farmers classifying this wheat as either spring or winter instead of facultative wheat (Braun & Sãulescu, 2002).

Facultative wheat is sown in autumn in these countries due to its rapid growth (shorter period for vernalisation) and higher frost tolerance than spring wheat.

Thus, facultative wheat grows and flowers earlier in spring compared to winter wheat (Braun, 1997; Carver, 2009; Monneveux et al., 2014).

Some wheat cultivars that have undergone vernalisation and experienced the appropriate photoperiod still display variations in their response to these factors. These variations are often attributed to earliness *per se* (*Eps*) genes, also referred to as ear emergence *per se*, intrinsic earliness, earliness in narrow sense, or as developmental rate genes (Ogihara et al., 2016; Huang et al., 2018).

Eps genes are distributed throughout the wheat genome and in related species; they are responsible for fine-tuning flowering time and maturity independently of the *VRN* and *PPD* genes. *Eps* genes can be defined as the minimum number of days to reproductive growth or the time to heading once vernalisation and photoperiod requirements have been satisfied (Ogihara et al., 2016; Huang et al., 2018).

For instance, kompetitive allele-specific PCR (KASP) markers have been used to genotype alleles of *Eps* genes in wheat landraces and cultivars from Pakistan. The KASP TaELF3-B1 marker, was used to identify alleles *Cadenza-type* and *Wild-type*, which are associated with variations in flowering times: late and early, respectively. These alleles are linked with the final number of spikes and grain number per spike (Zikhali & Griffiths, 2015; Ogihara et al., 2016; Huang et al., 2018; Iltaf et al., 2024).

Overall, the factors previously mentioned: vernalisation, photoperiod, and earliness *per se* genes, make wheat a highly adaptable crop, as it exhibits varied growth habits in response to different (and stressful) environmental conditions. Therefore, evaluating and classifying the performance of various wheat landraces (or varieties) under different conditions guide breeding programmes in developing resilient and high-yielding varieties adapted to specific climatic and agricultural areas, known as wheat mega-environments (MEs) (Reynolds & Braun, 2022).

1.5 Wheat Mega-Environments

Wheat mega-environments were defined by the International Maize and Wheat Improvement Centre (*Centro Internacional de Mejoramiento de Maíz y Trigo*, CIMMYT) as subregions spanning different countries sharing similar genotypic responses and best performing varieties (Rajaram et al., 1993; Yan et al., 2022). The genotypic characteristics encompass responses to various biotic and abiotic stresses, cropping-system requirements, water availability, temperature regimes, soil types, production systems, production volume, and consumer preferences (Rajaram et al., 1995; Ortiz et al., 2008).

For instance, wheat varieties adapted to high-rainfall regions exhibit distinct genetic backgrounds compared to those cultivated in arid or saline environments. This highlights the importance of accurately describing and understanding different MEs in order to develop breeding strategies designed to specific environmental conditions (Reynolds & Braun, 2022).

Twelve MEs (ME1 to ME12) have been identified for wheat (Figure 1.4), with six of them specifically dedicated to spring wheat cultivation areas: ME1 (irrigated, temperate), ME2 (high rainfall), ME3 (acid soils), ME4 (low rainfall), ME5 (high temperature), and ME6 (high latitude) (Rajaram et al., 1993).

Additionally, three MEs are dedicated to facultative wheat areas: ME7 (optimum environment, irrigated), ME8 (high rainfall), and ME9 (semi-arid). Lastly, three more MEs are characterised as true winter wheat areas: ME10 (optimum environment, irrigated), ME11 (high rainfall), and ME12 (semi-arid) (Rajaram et al., 1993).

These MEs provide valuable insights into crop land properties and help identify the most suitable wheat varieties for specific regions. Furthermore, they enable CIMMYT to monitor changes in these factors and track their effects on wheat production trends (Ortiz et al., 2008).

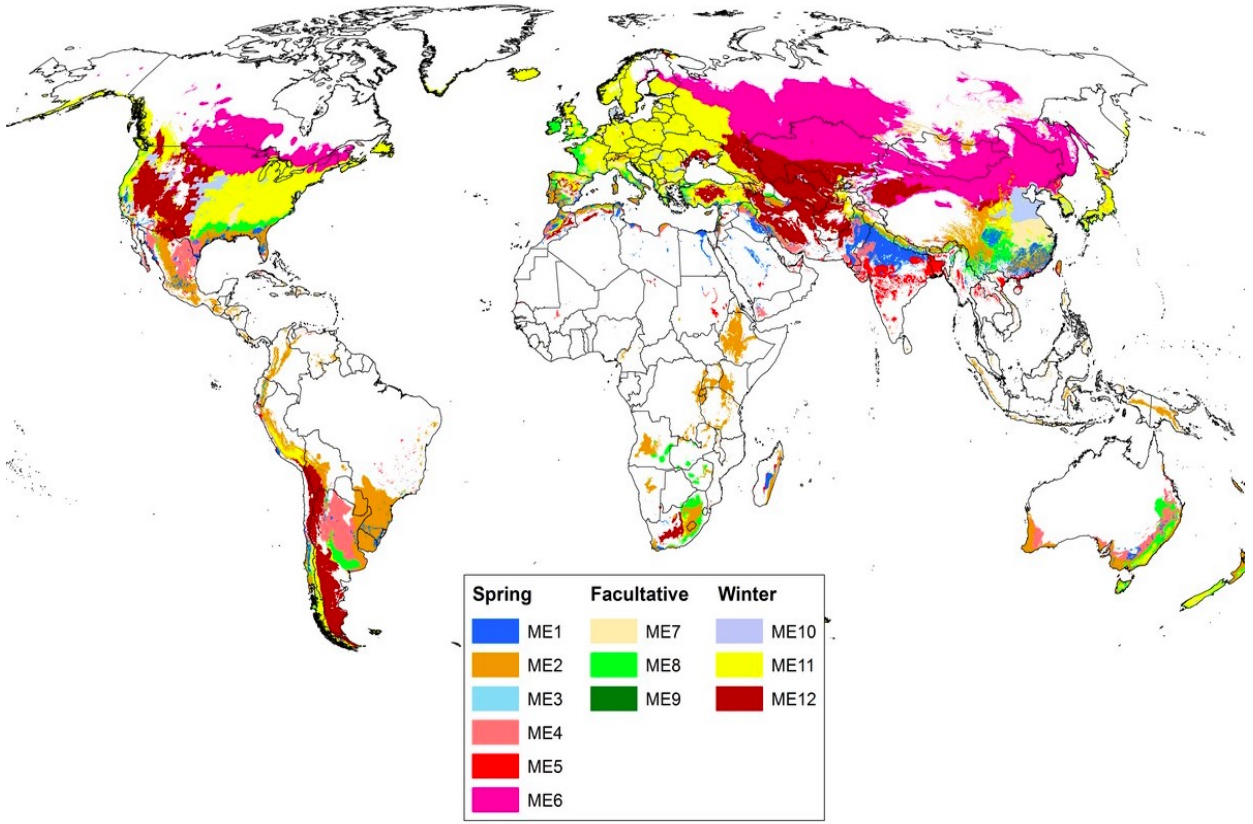


Figure 1.4 | Wheat Mega-Environments (MEs) worldwide. The map depicts 12 distinct MEs for spring, facultative, and winter wheat. Spring: ME1 (irrigated, temperate), ME2 (high rainfall), ME3 (acid soils), ME4 (low rainfall), ME5 (high temperature), and ME6 (high latitude). Facultative: ME7 (optimum environment, irrigated), ME8 (high rainfall), and ME9 (semi-arid). Winter: ME10 (optimum environment, irrigated), ME11 (high rainfall), and ME12 (semi-arid). Source: Sonder, 2016.

1.6 Wheat production

Triticum aestivum, the most widely cultivated wheat species, accounts for over 90% of global wheat production, serving as a staple for human consumption and, to a lesser extent, as livestock feed (Hawkesford et al., 2013). Moreover, besides its traditional uses, wheat grains have become an important source in liquid or gaseous biofuel production, particularly as a primary bioethanol feedstock in Europe. Additionally, wheat straw finds diverse applications, including arabinoxylan extraction (a dietary insoluble fibre) and particleboard manufacturing (Xu et al., 2019).

The global demand for wheat is increasing due to its use in the production of a wide range of essential and diverse food products (Shewry & Hey, 2015). Consequently, it is of vital importance to increase wheat production, ideally under a wide range of stressful conditions, with a primary focus on enhancing yields per unit area rather than expanding the cultivated land (FAO, 2009; Foresight Programme, 2011; Farooq et al., 2014). However, wheat cultivation has expanded across more land area worldwide than any other crop (Braun et al., 1997; Molnár-Láng et al., 2015).

This increase is expected to influence precipitation and temperature patterns, resulting in a higher frequency and intensity of drought stress in more than 50% of wheat cultivation areas (Farooq et al., 2014; Shayanmehr et al., 2020). Drought stress poses a significant challenge to global efforts to achieve food security, given the projected population growth to 9.7 billion by 2050. To meet the demand by 2050 and provide essential food for the growing population, crop production must increase by 60% (Hussain et al., 2016; Harkness et al., 2020; Camaille et al., 2021).

Climate change models project a substantial rise in the global average temperature by 0.5 to 3.7°C by the end of the year 2100. Jägermeyr et al. (2021) have reported a new systematic assessment of agricultural yield projections, based on previous projections from the Global Gridded Crop Model Intercomparison (GGCMI) and the Coupled Model Intercomparison Project Phase 5 (CMIP5). These collective multi-model simulations use collections of latest-generation crop and climate models,

which estimate how crops, including wheat, are expected to change as a consequence of climate change (Jägermeyr et al., 2021).

The outcomes include both losses and gains in crop yields (i.e., positive and negative responses) across various regions. Projections for wheat productivity suggest a relatively positive change globally, with losses in some cultivated areas (Figure 1.5). This positive response could be explained by enhanced plant growth due to a relatively stronger CO_2 response in wheat under certain conditions, principally when other stress factors are not present. Overall, this response in wheat leads to yield increases in high-latitude wheat MEs, but losses in some spring wheat MEs in Mexico, the southern United States, South America and South Asia (Jägermeyr et al., 2021).

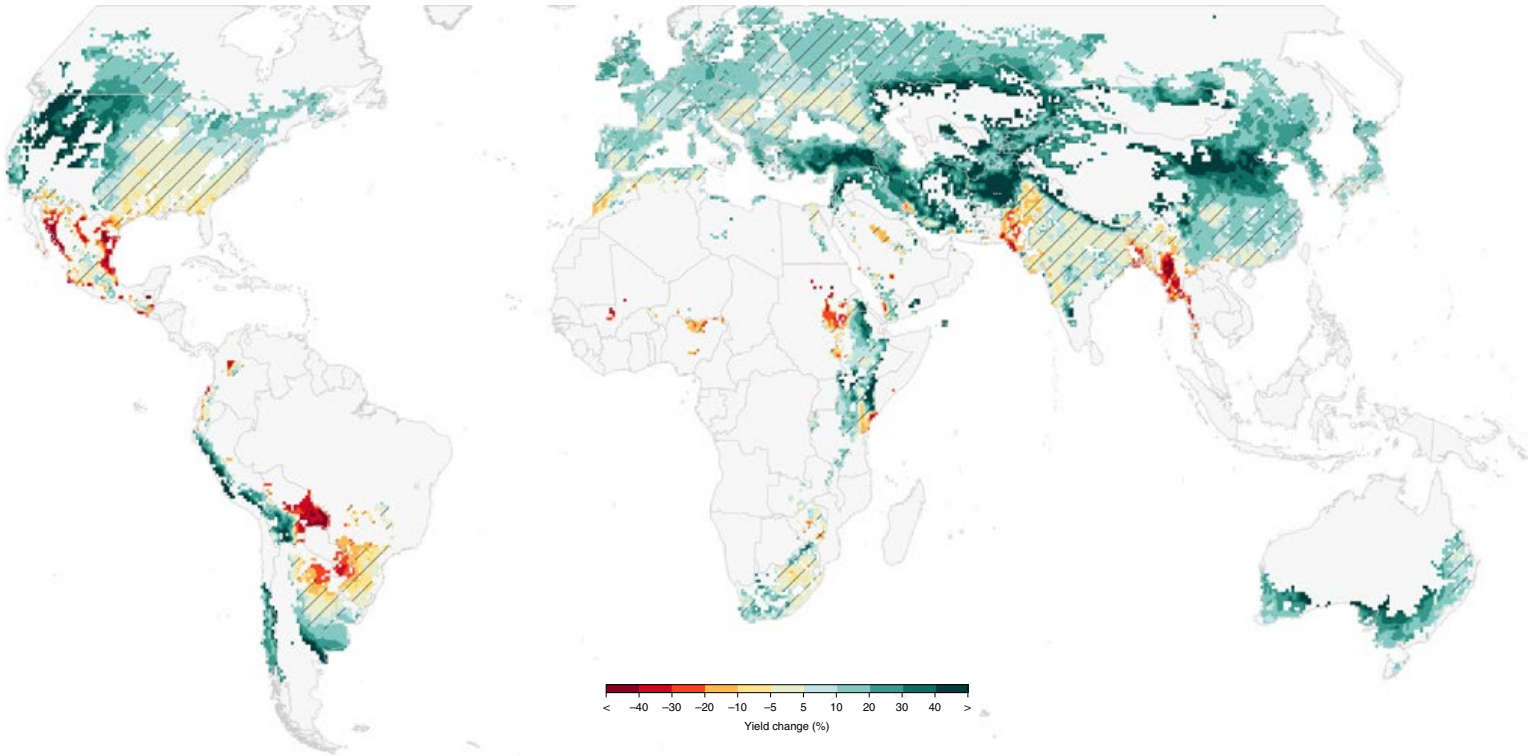


Figure 1.5 | Map showing median yield changes (period 2069–2099) for wheat across climate and crop models for current growing regions (>10 ha). Marking indicates areas where <70% of the climate-crop model combinations agree on the sign of impact. The percentage of the global production contributed by each zone is indicated by colour. Taken from Jägermeyr et al., 2021.

In the context of global cereal production, which includes wheat, maize (*Zea mays* L.), rice (*Oryza sativa* L.), sorghum (*Sorghum bicolor* L.), barley (*Hordeum vulgare*), and other grains, the total output amounts to 2,756 million tonnes, reflecting a two percent decrease compared to the previous year (FAO, 2022). Notably, wheat alone accounts for more than 700 million tonnes of this total production. Details on the main wheat-producing countries are referred to in Table 1.3.

Table 1.3 World wheat producers and wheat estimates from 2018 to 2020 (in million tonnes). Hyphens denote values summed up in "Other countries" category. Source: FAO, 2019 to 2022.

COUNTRY	2018	2019	2020
European Union (EU)	137.5	149.5	-
China (Mainland)	131.4	132.0	133.9
India	99.7	99.6	105.5
Russian Federation	72.1	82.0	80.1
United States of America (USA)	51.3	51.0	51.1
Canada	31.8	33.1	33.9
Pakistan	25.5	26.2	24.7
Ukraine	24.6	26.5	26.6
Türkiye	20.0	21.0	19.7
Australia	17.3	23.9	35.3
Argentina	19.5	19.8	22.6
Kazakhstan	13.9	14.0	-
Islamic Rep. of Iran	13.4	13.4	-
Other countries	72.2	75.0	241.7
Total	**730.2**	**767**	**775.6**

During 2018 and 2019, the European Union (EU) was the largest wheat producer, with France, Germany, the United Kingdom (UK), Spain, and Italy being the main contributors. However, since the UK left the EU in 2020, China (mainland) emerged as the leading producer, followed by India and the Russian Federation (FAO 2020; FAO, 2021; FAO, 2022).

On average, bread wheat production typically ranges from 2.8 to 3.5 tonnes per hectare (tonnes ha^{-1}). However, this yield is influenced by various biotic and abiotic factors (FAO/UN, 2012; Arendt & Zannini, 2013; Xu et al., 2019). For example, in

regions with dry environmental conditions like Australia and Mexico, grain yields are approximately 1 tonne ha^{-1}. Conversely, in some regions of the UK and France, the right combination of factors has led to yields of up to 10 tonnes ha^{-1} (Cracknell, 2015).

In addition to the main wheat species, smaller-scale production (10%) of other wheat species, such as durum (*T. durum*), spelt (*T. spelta*), einkorn (*T. monococcum*), and emmer (*T. dicoccum*), occurs in various regions worldwide. These species differ from *T. aestivum* in their physiological, morphological, and chemical composition, as well as their biochemical and clinical implications (Table 1.4) (Dinu et al., 2018).

Durum wheat accounts for approximately 5-6% of the total wheat production and is primarily used for pasta and bread production, as well as the production of semolina. A smaller portion of durum production (approximately one-sixth) is allocated for animal feed and various industrial applications (Collins et al., 2014; FAO, 2018).

The remaining 4 to 5% of total wheat production comprises spelt, einkorn, and emmer species. These three species were among the earliest cultivated; hence, they are referred to as ancient wheats. Spelt wheat is predominantly cultivated in mountainous regions of Europe, particularly in Germany and Switzerland. In the USA and Australia, spelt wheat is used to produce flour, primarily for making bread and beer (Cooper, 2015; Molnár-Láng et al., 2015).

Einkorn wheat, whose name is derived from the German word for 'single grain,' refers to the presence of a characteristic single grain per spikelet. Einkorn wheat continues to be cultivated in small areas in Türkiye, France, India, Europe, and North-Africa. Typically, it is grown under stressful soil conditions where other wheat cultivars may not survive. The food industry values einkorn for its rediscovered high nutritional benefits, which include a good balance of essential amino acids, minerals, and vitamins (Arendt & Zannini, 2013; Zaharieva & Monneveux, 2013; Pourkheirandish et al., 2018).

Emmer wheat is agriculturally important in central and northern Ethiopia, as well as in regions of India, Italy, Türkiye, and Iran, where it is widely utilised in the production of bread, pasta, and beer (Cooper, 2015). Its reputation as a healthy food source is also valued. This is primarily due to low glycaemic index and high satiety value exhibited by derivatives of emmer wheat, making it suitable for individuals with diabetes (Arzani & Ashraf, 2017).

Additionally, it possesses a lower quality of gluten (wheat proteins); the composition and content of gluten proteins contribute to the elasticity of the dough, a critical physicochemical characteristic in the production of breads and fermented derivatives. Since gluten can generate digestion-resistant peptides, a lower gluten quality facilitates digestion in susceptible individuals, such as those with celiac disease (Zaharieva et al., 2010; Arzani & Ashraf, 2017).

Table 1.4 Summary of key benefits of durum and ancient wheats (spelt, einkorn, and emmer) compared to bread wheat. Source: Hidalgo & Brandolini, 2013; Arzani & Ashraf, 2017; Dinu et al., 2018.

CATEGORY	DURUM	SPELT	EINKORN	EMMER	BREAD WHEAT
Protein content (%)	High (12-16)	High (12-15)	High (14-20)	High (14-18)	Moderate (12-14)
Amino acid profile	Moderate	Higher levels of essential amino acids, especially lysine			Moderate
Micronutrient content	Higher in minerals like zinc, iron, and magnesium; richer in B vitamins				Moderate, lower in comparison
Fibre Content	Moderate	Higher dietary fibre, particularly insoluble fibre			Moderate
Antioxidants	Moderate, higher than bread wheat	Higher levels of antioxidants, including carotenoids and phenolic compounds			Lower, compared to ancient wheats
Fatty acid composition	Higher in unsaturated fats than bread wheat	Moderate	High, particularly linoleic acid	Moderate	Lower, less unsaturated fats
Gluten	Strong, suitable for pasta making	Different gluten structure, often less elastic and potentially easier to digest			High elasticity, suitable for bread making, but problematic for some individuals
Glycaemic index	Lower glycaemic index, potentially beneficial for blood sugar management				Higher glycaemic index
Health benefits	Potentially greater health benefits due to higher nutrient content and bioactive compounds				Moderate, fewer benefits due to lower nutrient density

Although durum and ancient wheat are cultivated on a smaller scale, compared with bread wheat, diverse studies have investigated the effects of drought stress on them. Overall, durum wheat production is predicted to result in a decrease in yields of between 30 and 50% (Dettori et al., 2022). Research on spelt wheat has primarily focused on its drought tolerance mechanisms. However, mild drought stress (at 40% field capacity, FC) can cause significant damage to the plant (Martínez-Goñi et al., 2023).

Conversely, einkorn wheat has been utilised in breeding programmes as a valuable resource for alleles associated with resistance to various biotic and abiotic stresses, including drought, salt stress, leaf rusts, and powdery mildew (Yilmaz et al., 2022).

Emmer wheat is resistant to plant diseases and exhibits stronger tolerance to drought stress compared to spelt wheat, which is more sensitive to drought (Budak et al., 2013).

1.7 Mechanisms against drought stress in wheat

1.7.1. Drought stress

Drought stress is considered one of the most limiting factors that negatively affects plant growth, development, and production (Chen et al., 2018). In agriculture, drought is referred to as the insufficient availability of water from rainfall or by irrigation in the soil, which is needed by the crop. From a physiological perspective, the hydrated state of a cell, organ, or an entire plant is determined by water potential, a phenomenon that governs water movement (Buchanan et al., 2015).

Water potential is the sum of solute, pressure, gravitational, and matric potentials. Typically, the water potential is higher in the soil and lower in the roots, allowing water to diffuse from areas of high-water potential in the roots to nearby areas with lower water potential (Buchanan et al., 2015; Hussain et al., 2016).

Drought stress triggers multiple responses in plants, spanning from changes in gene expression and metabolism to alterations in developmental stages, i.e., vegetative and reproductive. These responses are influenced by factors such as plant species, the duration and severity of the drought, the affected organ or tissue, and interactions with other stressors. Nevertheless, plants cope with drought conditions by employing drought avoidance and/or tolerance strategies, which encompass morphological, physiological, and molecular adaptations (Buchanan et al., 2015; Hussain et al., 2016).

1.7.2. Plant signalling under drought stress

Under well-watered conditions, plants regulate their water status through the action of guard cells, which are highly specialised cells located on the lower surface of the leaf. These guard cells form tiny pores known as stomata. In response to environmental conditions, the guard cells undergo changes in shape, leading to the opening or closing of the pores (stomata). This mechanism permits the plant to control gas exchange and water transpiration (Lawson & Matthews, 2020).

When wheat plants sense drought conditions through complex signalling pathways, they initiate the synthesis of phytohormones in the plastids, predominantly abscisic acid (ABA), along with others such as ethylene, cytokinins, jasmonic acid, and auxins (Daszkowska-Golec & Szarejko, 2013). High concentrations of ABA delivered to the guard cells induce turgor changes through ion exchange, resulting in their shrinkage. This mechanical response provokes stomatal closure, preventing water loss (McAdam & Brodribb, 2018).

1.7.3. Reactive Oxygen Species and scavenging system

Nonetheless, prolonged stomatal closure reduces the entry of CO_2 from the atmosphere to the carboxylation site of Rubisco (ribulose 1,5 bisphosphate carboxylase/oxygenase, EC 4.1.1.39), an enzyme involved in carbon fixation. This reduction in CO_2 potentially results in the production of reactive oxygen species (ROS) (Das & Roychoudhury, 2014; Singh & Thakur, 2019).

Reactive oxygen species act as secondary messengers, triggering subsequent defence mechanisms to balance and neutralise the oxidative damage under normal growth and under stress conditions (Das & Roychoudhury, 2014; Singh & Thakur, 2019). The main types of ROS produced by plants under drought stress include free radicals such as superoxide ($O_2^{\bullet -}$) and hydroxyl radicals (HO•), as well as non-radicals like singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2), among others (Sachdev et al., 2021).

The ROS generated during drought stress can irreversibly damage cellular membranes and the photosynthetic machinery, which primarily comprises photosynthetic pigments such as chlorophyll a, chlorophyll b, pheophytins, and carotenoids, as well as photosystems PSI and PSII. This damage ultimately has a negative effect on the photosynthesis rate, leading to premature senescence of leaves and degradation of lipids, proteins, carbohydrates, and DNA (Demidchik, 2017).

However, plants have also evolved mechanisms to minimise the oxidative stress by generating non-enzymatic antioxidants like ascorbate (AsA), glutathione (GSH), tocopherol, flavonoids, carotenoids, and alkaloids, as well as enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione peroxidase (GPX). Non-enzymatic antioxidants play a role in maintaining the integrity of the photosynthetic membranes under stress, whereas the enzymatic antioxidants directly scavenge ROS or produce non-enzymatic antioxidants (Anjum et al., 2011; Demidchik, 2017).

Furthermore, wheat plants under drought stress accumulate osmoprotectants in the cytosol such as proline, sucrose, soluble carbohydrates, glycine betaine, and other organic solutes. These compounds contribute to maintaining the water potential in the plant cells (Yang et al., 2021).

As drought stress intensifies, wheat plants often increase proline accumulation, which helps to mitigate cellular damage by stabilising proteins and membranes, preserving their structure and function during dehydration. In addition to its role in osmotic adjustment, proline acts as a signalling molecule that can influence mitochondrial activity, regulate cell proliferation or death, and trigger the expression of stress-related genes, which are fundamental for plant recovery post-stress (Anjum et al., 2011).

1.7.4. Root and soil wheat microbiome

On a different level, under well-watered and drought conditions, the wheat root system regulates the root and soil microbiome (microbial communities) by releasing a diverse array of substances into the rhizosphere, the zone in the soil surrounding the roots. This process, known as rhizodeposition, has both direct and indirect effects on plant health and nutrition. The rhizodeposition also regulates diverse soil functions including nutrient availability and mobilisation, carbon sequestration, and soil aggregate formation (Curl & Truelove, 1986; Zhalnina et al., 2018; Williams & de Vries, 2020).

The released elements encompass sugars, organic acids, amino acids, vitamins, fatty acids, enzymes, secondary metabolites, border root cells, mucilage, and a variety of other compounds. Diverse factors influence the quantity and quality of the rhizodeposition (Figure 1.6), which consequently affect the microbial interactions in the rhizosphere (Curl & Truelove, 1986; Zhalnina et al., 2018; Williams & de Vries, 2020; Patra et al., 2021).

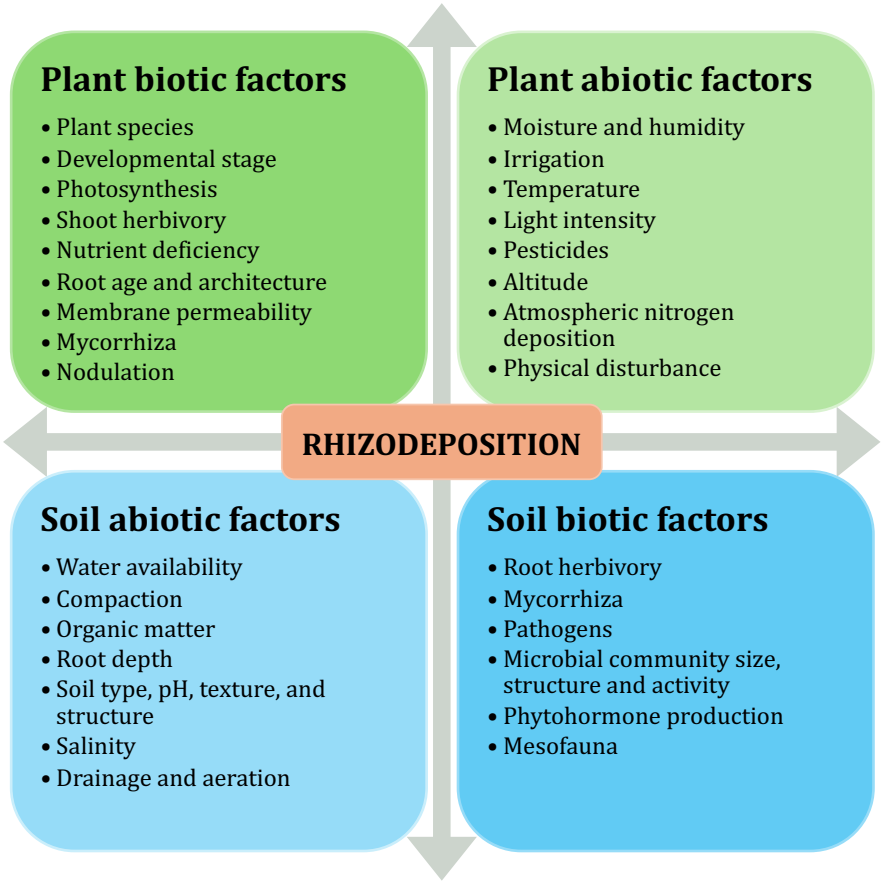


Figure 1.6 | Schematic representation of the biotic and abiotic factors of plant and soil that influence rhizodeposition. Modified from Patra et al., 2021.

Wheat root system transfers between 20 to 30% of total assimilated carbon into the soil, about 1.5 to 2 times less carbon compared with pasture plants (including grasses). Generally, most of this carbon will be used for root growth (7 – 13%), and around 7 to 14% is liberated by root respiration. Another part of this (2 – 4%), is adsorbed on clay minerals and soil organic matter (Kuzyakov & Schneckenberger, 2004; Patra et al., 2021).

Rhizodeposition only accounts for 1 to 2%; these carbon exudates are decomposed by microorganisms to CO_2 after being exuded into the rhizosphere, and approximately 0.8 to 3.2% is incorporated by rhizospheric microorganism (Kuzyakov & Schneckenberger, 2004; Patra et al., 2021).

The plant root system employs this strategy to attract and recruit beneficial microorganisms and build the wheat (root and soil) microbiome. Nevertheless, the

exact mechanism(s) underlying these interactions remain unknown (Lisar et al., 2012; Haichar et al. 2014; Zhalnina et al., 2018; Williams & de Vries, 2020; Patra et al., 2021). The beneficial interactions mainly include symbiotic associations with mycorrhizal fungi, but also associations with rhizobia, and plant growth promoting rhizobacteria (PGPR). However, plants also mediate negative interactions, including associations with parasitic plants, pathogenic microorganisms, and invertebrate herbivores (Lisar et al., 2012; Haichar et al. 2014).

1.8 Establishment of the AM symbiosis

The arbuscular mycorrhizal (AM) symbiosis is considered as the most important association between the roots of most land plant species, ranging from 70 to 90% and arbuscular mycorrhizal fungi (AMF) that belong to a range of taxa (Parniske, 2008; Bonfante & Genre, 2010; Smith & Read, 2010; Spatafora et al., 2016; Wipf et al., 2019; Rani et al., 2023).

AMF are ancient mutualistic symbionts that, based on fossil evidence, are estimated to have originated between 400 and 430 million years ago in tandem with the appearance of terrestrial plants (Parniske, 2008; Bonfante & Genre, 2010; Smith & Read, 2010; Spatafora et al., 2016; Wipf et al., 2019). Generally, the establishment of the AM symbiosis is divided into three phases (Figure 1.7): the asymbiotic, presymbiotic and symbiotic, which involve many morphological and physiological changes (Parniske, 2008).

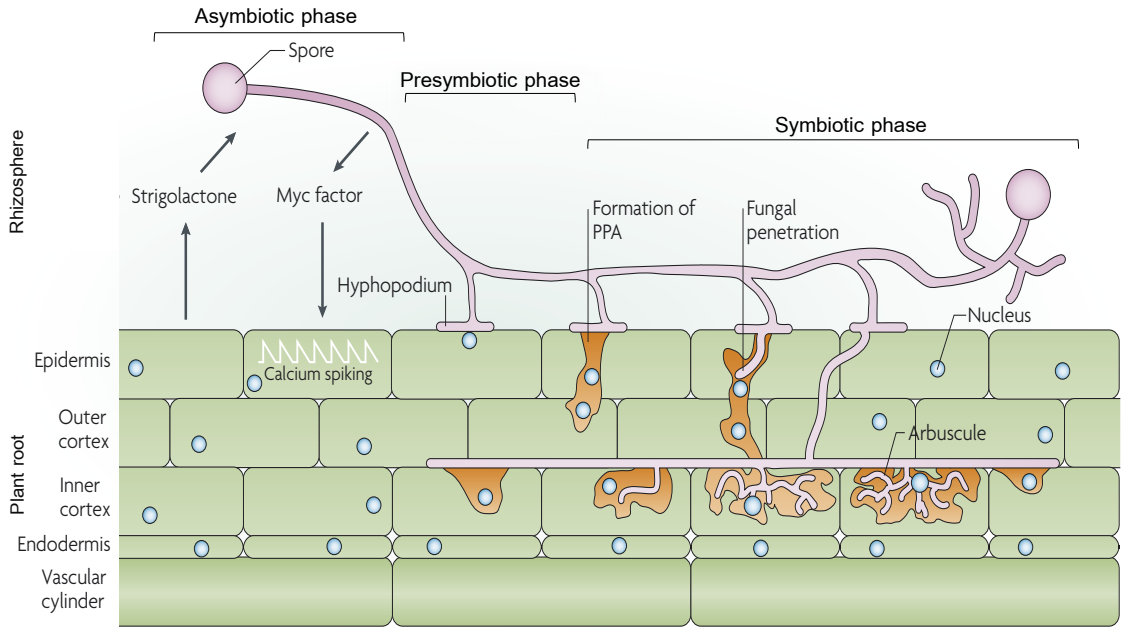


Figure 1.7 | Arbuscular mycorrhizal fungi (AMF) development can be divided into three phases: the asymbiotic, presymbiotic, and symbiotic. The first phase is characterised by the exudation of strigolactones by the roots, this phytohormone stimulates the germination and branching of the AMF spore. In response, the fungus releases chitin oligomers proposed as mycorrhization factors (Myc factors) for the activation of the symbiosis-signalling pathway. The second phase concludes with the development of the hyphopodium, a flattened hypha leading to the formation of the prepenetration apparatus (PPA). In the third phase, the hypha penetrates the cortical cells through the PPA, and in the inner cortex, it develops an arbuscule for nutrient exchange. Modified from Parniske, 2008.

In a broader context, the initial phase involves spore germination, during which hyphae extend towards the plant roots. The second phase activates the symbiosis-signalling pathway, promoting further hyphal growth. Finally, in the third phase, the symbiosis is established, where, depending on the AMF colonisation strategies and the selective factors employed by the plant, the fungus enters and colonises the plant roots (Malbreil et al., 2014; Cormier et al., 2016; Hugoni et al., 2018).

This process facilitates the exchange of water and mineral nutrients to the plant, in return, the plant supplies a carbon source and a niche for the completion of the fungal life cycle through sporulation (Parniske, 2008; Smith & Read, 2010; Malbreil et al., 2014; Cormier et al., 2016; Hugoni et al., 2018).

Several studies have shown that AMF play a significant role in agroecosystems, particularly in cereal cultivation (Al-Karaki et al., 2003; Talaat & Shawky, 2014; Bernardo et al., 2019; Duan et al., 2020; Meier et al., 2022). Over time, studies have described seven types of mycorrhizae, but some are restricted to specific plant families (Table 1.5). Conventionally, four main types are considered: arbuscular mycorrhiza (AM), ectomycorrhiza (EcM), ericoid mycorrhiza (ErM), and orchid mycorrhiza (OrM) (Brundrett, 2002; Brundrett & Tedersoo, 2018).

Table 1.5 General characteristics of the main mycorrhizal types. Modified from Smith & Read, 2010; Brundrett & Tedersoo, 2017.

MYCORRHIZAE	MORPHOLOGICAL CHARACTERISTICS			BENEFITS TO PLANTS	BENEFITS TO FUNGI	PLANT ASSOCIATIONS	FUNGAL ASSOCIATIONS
	FUNGAL STRUCTURES	HYPHA TYPE	INTRA-CELLULAR COLONISA-TION				
Arbuscular	Arbuscules present; vesicles present/absent. Colonisation from root surface mycelia or from neighbouring cells	Aseptate	Yes	Nutrition (mineralised nutrients), limited protection	Carbon energy, habitat, deep water from trees	Angiosperms Gymnosperms Bryophytes Pteridophytes	Glomeromycotina
Ectomycorrhizal	Hartig net present; differentiated hyphal mantle present	Septate	No	Nutrition (mineralised, simple organic nutrients), protection	Carbon energy, deep water from trees	2 Gymnosperms and 28 Angiosperms lineages	Basidiomycota Ascomycota
Ericoid	Hyphal coils in cells present; each cell is separately colonised from root surface	Septate	Yes	Nutrition (mineralised, simple organic nutrients), limited protection?	Carbon energy? Habitat in roots and soil via	Ericales Bryophytes	Ascomycota
Orchid	Hyphal pelotons present; old pelotons digested by plant. Colonisation from root surface mycelia or from neighbouring cells	Septate	Yes	Nutrition (mineralised, organic nutrients, carbon energy), limited protection?	None (probably cannot support fungi)	Orchidales	Basidiomycota

1.9 AMF classification

When first classified, AMF were grouped in the Zygomycota phylum as part of the Endogonales order, primarily based on their morphology, and those lacking defined characteristics were often default-grouped into the genus *Glomus*. However, through the use of the small subunit ribosomal RNA (SSU rRNA), a conserved region, the AMF were reclassified into the Glomeromycota phylum, which contained the Glomerales, Diversisporales, Archaeosporales and Paraglomales clades (Figure 1.8) (Young, 2015).

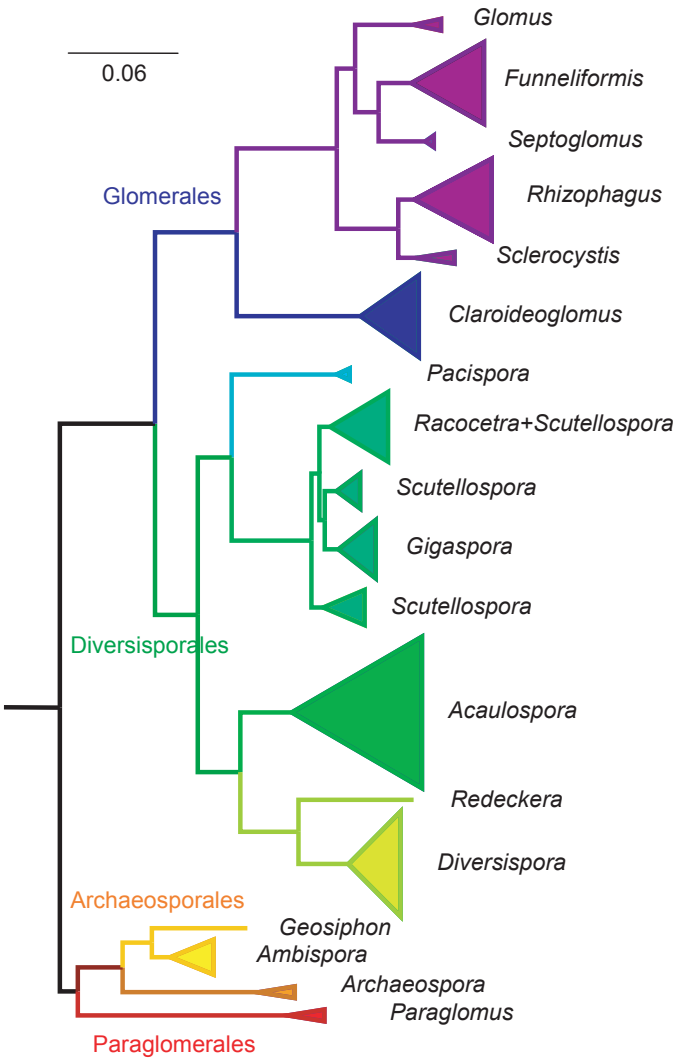


Figure 1.8 | Phylogenetic relationships between members of the Glomeromycota phylum based on the small subunit ribosomal RNA (rRNA) sequences. The phylum is formed by four clades, namely the Glomerales, Diversisporales, Archaeosporales and Paraglomales. Taken from Young, 2012.

More recently, with the availability of advanced molecular techniques and technologies, AMF have been further classified into a separate subphylum, the Glomeromycotina, which is the sister taxon to the Mortierellomycotina within the phylum Mucoromycota (Young, 2015; Spatafora et al., 2016; De Leon et al., 2020).

All members of this subphylum share the characteristic of forming AM, except for *Geosiphon*, which is a symbiont of cyanobacteria. AMF are widespread and found in virtually all ecosystems, although their diversity is relatively low, with more than 240 recognised species. It is estimated, however, that a larger number exists (Spatafora et al., 2016; Kim et al., 2017).

The first AMF *in vitro* root organ culture was determined as *Glomus intraradices*, currently, *Rhizophagus irregularis* (syn. *Glomus irregulare or Rhizophagus intraradices*), and descendants resulting from this culture are referred to as *G. intraradices* DAOM197198 or DAOM181602 (Stockinger et al., 2009). This particular AMF species have been extensively used in different trials involving agriculturally important plants, including wheat; thus, it was considered a model organism within the AMF, being the first to be sequenced (Stockinger et al., 2009; Mathieu et al., 2018; Walker et al., 2021b; Wendering & Nikoloski, 2022).

AMF are obligate biotrophs, meaning they require a host plant to complete their life cycle. The biotrophic nature of AMF remains a conundrum, but it has been proposed that it is influenced by nutritional, physiological, and genetic aspects (Wipf et al., 2019). Sexual reproduction of AMF is not yet known, but asexual reproduction occurs through spores, which can be found resting in the soil in a wide range of environments such as grasslands, croplands, forests, deserts, dunes, as well as alpine and boreal zones (Redecker et al., 2013).

Due to their asexual and clonal nature, the concept of AMF species is controversial, as the biological species concept is difficult to apply (Redecker et al., 2013). Additionally, the large genetic variation observed within a single AMF species could suggest that these variations represent either distinct species or intra-species diversity (Rosendahl, 2008). However, they can still be referred to as species or

morpho-species, with the latter based on the morphological characteristics of the spores (Wipf et al., 2019).

1.10 Exchange of resources from the AMF to the plant

1.10.1. Water transport by AMF

Water uptake in plants occurs by a difference in the water potential between the roots and the soil, and its movement inside the plant is mediated by membrane water channels or aquaporins (AQPs) (Ahmed et al., 2020). Aquaporins are present in virtually all organisms, where they facilitate the transport of water and small molecules across membranes. In plants, AQPs are classified into seven groups, while in fungi, there are five. Fungal AQP groups are further subdivided into two classical AQPs and three groups of aquaglyceroporins (permeable to small, uncharged molecules, such as glycerol and ammonia) (Li et al., 2013; Ahmed et al., 2020).

When the symbiotic relationship is established with the plant, the AMF may regulate water loss by upregulating root AQP genes to increase water uptake or downregulating leaf AQP genes to decrease the leaf transpiration rate when the plant is under drought stress (Li et al., 2013; Kakouridis et al., 2022).

Despite the established knowledge that the extraradical hyphal network can access soil micropores as small as 2 to 30 μm in diameter to uptake water and transport it directly to the roots, it was previously believed that the amount was insufficient to cope with drought stress. However, a recent study by Kakouridis et al. (2022) utilised isotopically labelled water ($H_2{}^{18}O$) and a membrane-impermeant fluorescent dye in a microcosm to irrigate and directly quantify the water transported by the AMF species *R. irregularis* in *Avena barbata* plants (Figure 1.9) (Bárzana et al., 2012; Püschel et al., 2021).

This work strongly suggests that in mycorrhizal plants, AMF not only transport water through their extraradical hyphae but also extracytoplasmically within the

hyphae, indicating an enhancement in the plant-water relationship and its potential role in improving tolerance to drought stress (Kakouridis et al., 2022).

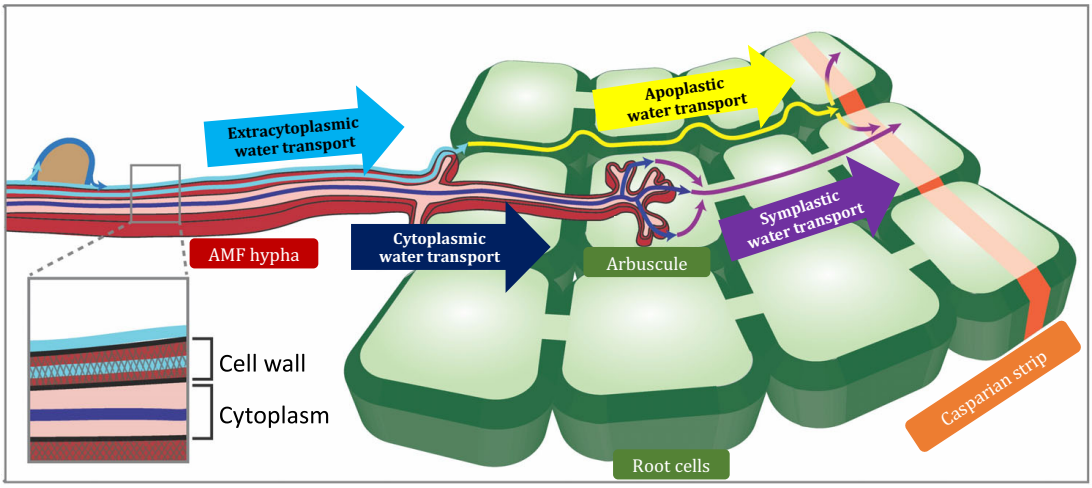


Figure 1.9 | Representation of water transport from the soil through an arbuscular mycorrhizal fungus (AMF) hypha to a plant root. Extracytoplasmic water transport in a hypha (light blue arrow) joins the apoplastic transport in a plant root (yellow arrow), while cytoplasmic transport in a hypha (dark blue arrow) joins the symplastic transport in a plant root (purple arrow). Modified from Kakouridis et al., 2022.

These findings align with those reported by Wu et al. (2024), who utilised a compartmentalised system to investigate the role of AMF in water transport. In their study, the AMF hyphae were allowed to grow into a separate section containing ^{18}O-labelled water, while the plant roots were confined to a different division. This setup enabled the researchers to directly measure the amount of water transported by the AMF hyphae from the water compartment to the plant roots (Wu et al., 2024).

The results showed that the water transported by the AMF hyphae was significant and served as a supplementary water source for the plants, particularly under conditions where the roots alone could not access sufficient water. The study highlighted that while the direct contribution of AMF hyphae to water transport might be relatively limited, this mechanism is crucial during periods of drought stress, where every additional source of water can increase plant survival chances and growth (Wu et al., 2024).

On the other hand, AMF can also play a crucial role in facilitating water transport between plants via common mycorrhizal networks (CMNs). In a study by Egerton-Warburton et al. (2007), an experiment was conducted where different plants were connected through CMNs, i.e., one group of plants accessed a deeper, deuterium-enriched water source, while the other group was situated in drier soil.

The findings revealed that AMF facilitated the movement of water from the well-watered plants to the drought-stressed ones, effectively redistributing water among neighbouring plants during drought conditions. This process enhanced the water status as long as CMNs were maintained. This study highlights the ecological significance of AMF in supporting plant survival and resilience in environments with limited water availability (Egerton-Warburton et al., 2007).

1.10.2. Plant mineral nutrients

For homeostatic balance in the plant, a range of mineral nutrients, classified as macro- and micronutrients, are needed at different plant tissues and at concentrations to complete its life cycle. The macronutrients, namely carbon, hydrogen, oxygen, nitrogen, phosphorus, potassium, calcium, magnesium, and sulfur, are required in large quantities, ranging from 1 to 15 mg per gram of dry weight (Buchanan et al., 2015; Hasanuzzaman et al., 2018).

Nitrogen, phosphorus, and potassium are classified as primary mineral nutrients, while calcium, magnesium, and sulfur are classified as secondary mineral nutrients. Conversely, micronutrients, including iron, manganese, chlorine, zinc, copper, boron, molybdenum, and nickel, are required in smaller quantities, typically 100 to 10,000 times less than macronutrients (Buchanan et al., 2015; Hasanuzzaman et al., 2018).

Imbalances in minerals caused by abiotic conditions, such as drought stress, can profoundly affect plant growth, development, and production. Consequently, plants have evolved various mechanisms to mitigate the effects of both deficiencies and excesses of these macro- and micronutrients (Buchanan et al., 2015).

1.10.3. Phosphorus

Phosphorus is vital for plant growth and development, playing crucial roles in photosynthesis, respiration, cell membrane formation, enzymatic activities, root development, and seed formation. Moreover, phosphorus is present in the form of soluble inorganic phosphate anions (P_i), specifically dihydrogen phosphate ions ($H_2PO_4)^{-1}$ and orthophosphate ($HPO_4)^{-2}$, which are transported into the root cells from the soil (Buchanan et al., 2015; Billah et al., 2019; Meier et al., 2022).

Additionally, phosphorus exists in organic forms within plants, such as adenosine diphosphate (ADP), adenosine triphosphate (ATP), nucleotides, phospholipids, phosphoproteins, nucleic acids, and coenzymes (Buchanan et al., 2015; Billah et al., 2019; Meier et al., 2022).

On the other hand, due to its low availability under most soil conditions, phosphorus is a restricted mineral nutrient, resulting in a greater uptake rate than its renewal. This phosphorus deficiency in the soil can lead to negative physiological and morphological symptoms, including chlorosis, necrosis, and an overall reduction in growth and development in the plant. This ultimately limits crop yield (Buchanan et al., 2015; Billah et al., 2019).

Furthermore, due to the rapid depletion of phosphorus from the rhizosphere by the roots, plants employ various morphological, physiological, and molecular mechanisms to avoid P_i starvation (Varma et al., 2017; Hasanuzzaman et al., 2018; Wu et al., 2018; Cataldi et al., 2020). These mechanisms include I) modifications in root architecture and branching, II) an increase in root hair density, III) induction of high-affinity P_i transporters (PHTs), IV) release of organic acids for solubilisation, such as citric, malic, and oxalic acids, V) release of enzymes such as phosphatases, phytases, and carbon-phosphorus lyases, and VI) association with beneficial microorganisms, such as PGPR and AMF (Varma et al., 2017; Hasanuzzaman et al., 2018; Wu et al., 2018; Cataldi et al., 2020).

However, the mechanisms to avoid P_i starvation may vary between plant species or even between cultivars due to factors such as phosphorus demands, root system characteristics, mycorrhizal dependency, among others (Varma et al., 2017; Hasanuzzaman et al., 2018; Wu et al., 2018; Cataldi et al., 2020).

1.10.4. P_i starvation and AMF

The uptake of phosphate ions by the plant primarily occurs in the root tips through diffusion. However, this process is delayed by the slow-moving nature of phosphate ions in the soil, where the concentration typically ranges between 0.1 to 10 μM. Soil concentration is lower than that within the plant, which is approximately 5 to 20 μM. Consequently, the transport of P_i is dependent on the water content in the soil surrounding the roots. In regions characterised by arid, semi-arid, or drought-prone conditions, the availability of P_i is low, thus limiting its absorption by the plant (Püschel et al., 2021).

The difference in P_i concentration between the plant roots and the soil, leads to active transport into cells through energy-driven P_i-H^+ co-transporters. This process operates against the concentration gradient and involves the utilisation of an electrochemical potential gradient generated by H^+-ATPases (Johri et al., 2015; Hasanuzzaman et al., 2018).

Phosphate transporters are classified into four gene families: PHT1, PHT2, PHT3, and PHT4. The PHT1 family is the largest and is primarily located in the plasma membrane of roots (Gu et al., 2016). Fourteen PTH1 genes have been reported in wheat (denominated TaPTH1 1-14), located on different chromosomes. Interestingly, these genes were found to be expressed during various developmental stages and were highly induced in roots colonised by specific AMF species (Victor et al., 2019).

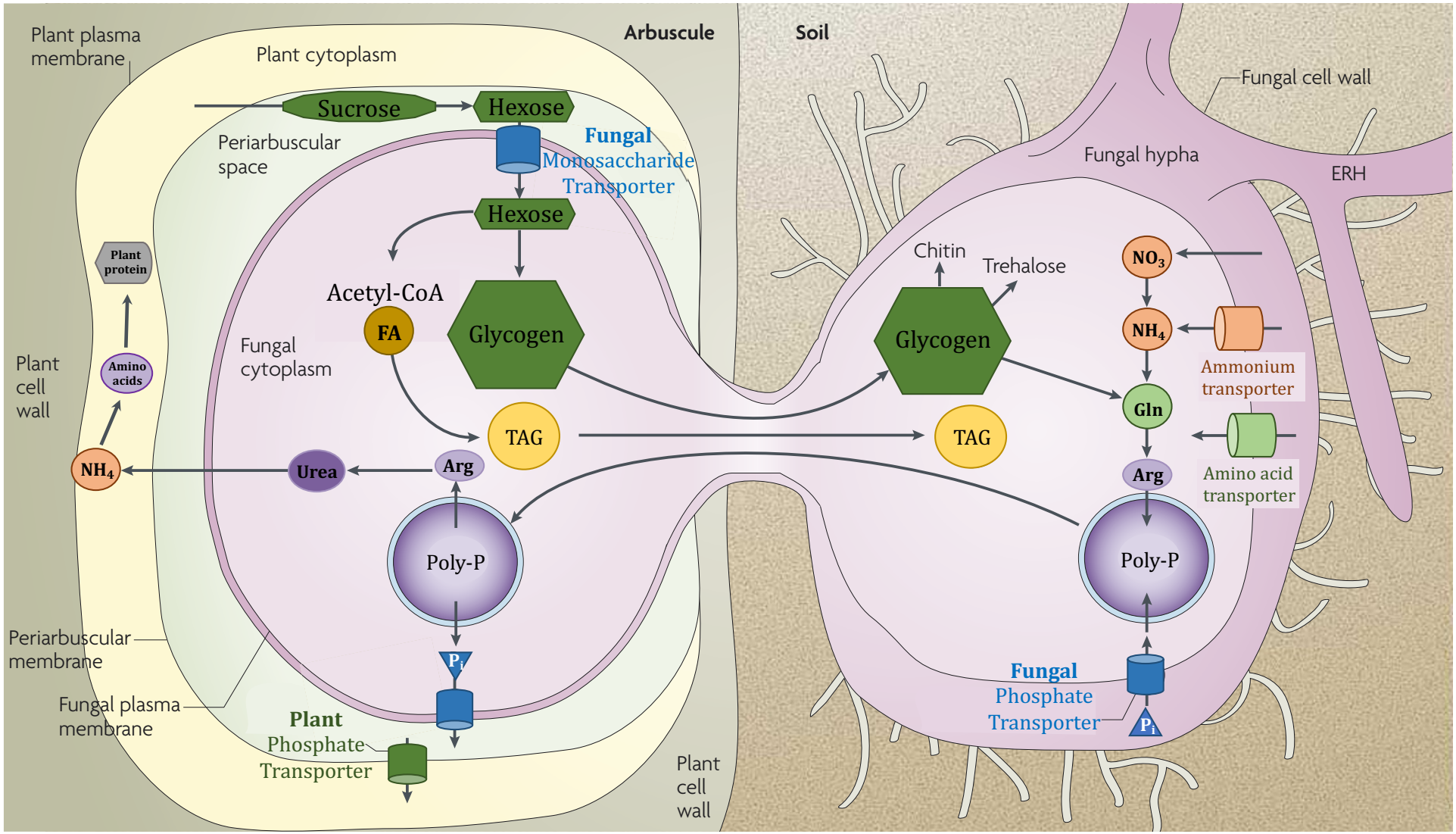
Active adsorption of inorganic phosphate by the plant results in a P_i-depletion zone. When no phosphate is available, the plant experiences P_i starvation. In response to this limitation, plants have the ability to associate with beneficial microorganisms,

predominantly forming associations with AMF (Ferrol et al., 2019; Kobae, 2019). Extraradical AMF hyphae can access soil pores and extend further from the P_i-depletion zone, up to 25 cm away from the roots, resulting in more efficient mineral nutrient uptake (Smith & Smith, 2011).

In the context of the symbiotic relationship, P_i concentration plays a critical role. High levels of P_i in the soil can temporarily suppress or inhibit key aspects of AMF development, including spore formation, intraradical colonisation, and the development of arbuscular structures; these effects continue until P_i concentrations decrease (Ferrol et al., 2019; Kobae, 2019).

Moreover, it has been demonstrated that the association with AMF leads to the downregulation of plant PHT genes responsible for direct P_i uptake in the root system. Consequently, the majority of P_i in the plant is absorbed and translocated by the fungal PHTs located along its extraradical hyphae, which function similarly to those in plants (Kobae et al., 2016; Lanfranco et al., 2018). Moreover, there is evidence that the extraradical hyphae of the AMF species *Rhizophagus clarus* are capable of solubilising organic phosphorus by releasing a phosphatase into the substrate (Sato et al., 2019).

Once inside the extraradical hyphae, the adsorbed P_i is converted into polyphosphate chains (poly-P), which are linear chains with hundreds of P_i molecules. These chains are then translocated to the intraradical hyphae, where they are hydrolysed to liberate the P_i from the polyphosphate chains (Figure 1.10). Finally, the P_i is released into the PAS by an unknown mechanism. Subsequently, P_i is imported into the arbusculated cortical cells by another group of plant transporters (Johri et al., 2015; Kobae et al., 2016; Varma et al., 2017; Lanfranco et al., 2018; Victor et al., 2019).


Plant plasma membrane
Plant cytoplasm
Arbuscule
Soil
Fungal cell wall
Fungal hypha
ERH
Sucrose
Hexose
Periarbuscular space
Fungal Monosaccharide Transporter
Hexose
Plant protein
Amino acids
Plant cell wall
Acetyl-CoA
FA
Glycogen
Fungal cytoplasm
Chitin
Trehalose
Glycogen
NO_3
NH_4
Ammonium transporter
Gln
Amino acid transporter
Arg
TAG
TAG
NH_4
Urea
Arg
Poly-P
Poly-P
P_i
Periarbuscular membrane
Fungal plasma membrane
Plant Phosphate Transporter
Fungal Phosphate Transporter
P_i
Plant cell wall

← **Figure 1.10 |** Uptake of mineral nutrients and exchange of resources between the plant and arbuscular mycorrhizal fungi involve several processes. Inorganic phosphate anions (P_i) in the soil are imported by a fungal phosphate transporter located in the extraradical hyphae. P_i is transported in the form of polyphosphate granules (Poly-P) into the intraradical hyphae and subsequently released into the periarbuscular space of the arbuscule, facilitating transport to the cortical cells of the plant. Nitrogen, in the form of nitrate (NO_3^-) or ammonium (NH_4), or in the organic form of amino acids (arginine, Arg or glutamine, Gln), is translocated from the soil through high and low-affinity fungal transporters located on the extraradical hyphae. Nitrogen is released from arginine in the form of urea in the arbuscule and then transported to the cortical plant cells. On the other hand, plant-derived carbohydrates, in the form of sucrose, are transported first to the periarbuscular space with a high-affinity monosaccharide transporter, then to the arbuscule in the form of hexoses. These hexoses are metabolised into lipids such as fatty acids (FA), which are the main components of phospholipids and neutral lipids such as triacylglycerol (TAG) to be used by the fungus. Modified from Parniske, 2008.

1.10.5. Nitrogen

Nitrogen is one of the most important mineral nutrients for living organisms. Approximately 80% of nitrogen is found as atmospheric dinitrogen (N_2). In plants, nitrogen comprises around 1 to 15% of their dry weight. It is present in diverse plant tissues as organic molecules, including nucleic acids, proteins, chlorophyll, hormones, and derivatives from nitrogenous precursors and secondary compounds (Buchanan et al., 2015; Jiang et al., 2019).

The complexity of the nitrogen cycle is due to the many oxidation states of nitrogen atoms, which generate multiple organic and inorganic forms (Buchanan et al., 2015). These forms constitute less than 0.1% of nitrogen in the soil, with their availability varying depending on a broad range of physicochemical characteristics, including pH, as well as environmental conditions such as temperature and water content. Therefore, nitrogen is considered a limiting factor for plant development and crop yield (Buchanan et al., 2015; Jiang et al., 2019). For instance, well-aerated soils are correlated with high levels of nitrate (NO_3^-), whereas in anoxic or acidic conditions, nitrate levels are low, and ammonium ion (NH_4^+) levels are higher (Foyer & Zhang, 2011; Buchanan et al., 2015).

Most of the nitrogenous forms result from diverse processes such as nitrification, denitrification, anaerobic ammonium oxidation, and nitrogen fixation, which are exclusively carried out by a wide variety of rhizospheric microorganisms, including free-living nitrifying rhizobacteria or endosymbionts (bacteria and fungi). These microorganisms can use different oxidised forms of nitrogen to produce other nitrogenous compounds, which, in turn, can be taken up or recycled by other microorganisms (Buchanan et al., 2015; Jansa et al., 2019).

1.10.6. Nitrogen transport in plants

All studied plant species have the inherent capacity to uptake nitrogen; however, they show a preference for organic nitrogen compounds, particularly amino acids, namely arginine, alanine, glutamate, glutamine, and ornithine, with arginine being the most frequently absorbed. Following the uptake of organic forms, plants absorb inorganic nitrogen primarily as ammonium ions and nitrate, respectively (Di Martino et al., 2018; Gibelin-Viala et al., 2019).

Under optimal nitrogen concentrations in the soil, plant roots are self-sufficient in absorbing most nitrogenous forms. However, when passive transport is limited or needs adjustment, plants utilise active transport systems involving ammonium transporters (AMT) or nitrate transporters (NTR) (Ludewig et al., 2007; Wu et al., 2021).

The NH_4^+ transporters are classified within the AMT family. In wheat, at least 54 AMT homologs have been identified. The AMT are divided into two subfamilies: AMT1 and AMT2. The former is highly expressed in roots compared to above-ground tissues, whereas the latter is commonly expressed at low levels in roots, shoots, and leaves (Jiang et al., 2019).

The NO_3^- transporters are classified into two transport systems: the low-affinity transport system (LATS) and the high-affinity transport system (HATS). The LATS is included in the nitrate transporter 1 or nitrate peptide transporters (NRT1/NPF) family, while the HATS belongs to the nitrate transporter 2 (NRT2) family. However,

some transporters show dual affinity (Wu et al., 2021; Barzana et al., 2021; Wang et al., 2022).

1.10.7. Nitrogen uptake by AMF

When nitrogen concentration is suboptimal in the soil, plants commonly form associations with beneficial microorganisms, including AMF. Under these conditions, AMF play a significant role in supporting the plant. The AMF explore areas beyond the nutrient depletion zone using their extraradical hyphae or mycelium. It is suggested that AMF preferentially adsorb NH_4^+ over NO_3 directly from the soil, as the former is energetically more efficient. Following this, they absorb organic nitrogenous forms, such as amino acids, peptides, chitin oligomers, and nucleotides (Wu et al., 2021).

AMF have the ability to decompose organic matter by releasing peptidases and proteases into the soil to obtain nitrogen in the form of peptides, proteins, or free amino acids, which are subsequently transported to the plant. However, it has been suggested that AMF prioritise their own nitrogen demands for development, and only after fulfilling these needs they benefit the plant. It is expected that AMF use nitrogen transporters, mechanisms, and pathways similar to those in plants to uptake nitrogen from the soil (Hodge, 2001; Varma et al., 2017; Jansa et al., 2019).

The first step in nitrogen uptake is the same as the one carried out by the plant: through the action of the nitrate reductase, as the name implies, it reduces NO_3^- to NH_4 (Figure 1.10). Secondly, glutamine synthetase (GS) catalyses the conversion of ammonium (NH_4) into the amino acid glutamine (Gln). In the species *R. irregularis*, two GS isoforms have been identified: GiGS1 and GiGS2. Both are expressed in the extraradical hyphae, but the latter is expressed when NO_3^- is high (Govindarajulu et al., 2005; Di Martino et al., 2018; Devanna et al., 2021; Howard et al., 2022).

Thirdly, via the glutamine-2-oxoglutarate amino-transferase (GOGAT) pathway, Gln undergoes transformation, ultimately resulting in the formation of the amino acid arginine (Arg) (Govindarajulu et al., 2005; Di Martino et al., 2018; Devanna et al.,

2021; Howard et al., 2022). Subsequently, the Arg produced within the extraradical hyphae through the GS-GOGAT pathway is translocated to the intraradical hyphae, where additional enzymatic processes transform Arg into urea for direct transfer to the plant (Parniske, 2008; Basu et al., 2018).

1.10.8. Measurement of nitrogen status in plant leaves

As mentioned above, nitrogen is a component of chlorophyll content since chloroplasts within leaf cells hold approximately 70% of the nitrogen in leaves. The determination of the chlorophyll content in crop plants, directly or indirectly provides valuable information for plant nutritional status, nitrogen fertiliser management, plant growth, vigour, etc (Song et al., 2021).

Greenness leaf colour is an indicator of the plant chlorophyll content, reflecting the plant nitrogen status. Moreover, chlorophyll content may be increased or decreased under environmental stresses, including drought stress. Therefore, the measurement of leaf chlorophyll content would provide information related to photosynthesis in wheat landraces and changes correlated with its potential drought tolerance (Mamrutha et al., 2017; Song et al., 2021).

Various methods can be utilised to determine the chlorophyll content, ranging from traditional laboratory techniques to advanced non-destructive tools. These methods differ in their accuracy, complexity, and suitability for field or laboratory use. Below, Table 1.6 summarises methods for detecting chlorophyll in crop plants, including wheat, highlighting their principles, advantages, and disadvantages (Samsone et al., 2007; Novichonok et al., 2016; Mulero et al., 2022).

Table 1.6 Overview of various chlorophyll detection methods in crop plants, highlighting their principles, advantages, and disadvantages. Source: Samsone et al., 2007; Saberioon et al., 2014; Novichonok et al., 2016; Mulero et al., 2022.

METHOD	PRINCIPLE	ADVANTAGES	DISADVANTAGES
- Destructive:			
SPECTROPHOTOMETRY	Chlorophyll is extracted from plant tissues (usually leaves) using solvents. The absorbance of the extract is measured at specific wavelengths.	- High accuracy and precision. - Can differentiate between chlorophyll a and chlorophyll b, as well as carotenoids without prior separation.	- Requires destruction of plant tissue. - Time-consuming. - Use of hazardous chemicals. - Minimal time lapses between the sample collection and their analysis since pigments break down quickly.
- Non-destructive:			
PORTABLE CHLOROPHYLL METRES	Devices like SPAD and atLEAF CHL metres estimate chlorophyll content by measuring the leaf greenness through a transmission of light.	- Allows repeated measurements on the same plants. - Fast and easy to use in the field. - Measure difference in absorbance at two wavelengths.	- Provides relative chlorophyll content, not absolute. - Accuracy can be influenced by factors, such as leaf thickness, water content, and light intensity.
REMOTE (FLUORESCENCE) IMAGING	Uses satellite or drone-based sensors to measure reflectance data related to chlorophyll content and photosynthetic efficiency.	- Provides immediate results for real-time monitoring. - Capable of monitoring large (field) areas. - Can provide multi-temporal data for tracking changes over time.	- Expensive equipment. - Lower accuracy compared to ground-based methods, requires calibration and validation data. - Sensitivity to environmental factors, such as light intensity and temperature. - Requires technical knowledge for equipment use and data interpretation.

Traditional measurement of chlorophyll in wheat involves extraction of plant tissues (leaves) with solvents like acetone, dimethyl sulfoxide (DMSO) or by N, N-Dimethylformamide, with subsequent spectrophotometric measurement of the absorbance. This method involves destroying samples, which is undesirable for phenotyping wheat (Mamrutha et al., 2017).

Various studies have been assessed using this method to monitor chlorophyll *a* and *b* levels in wheat landraces and cultivars in different environments, demonstrating a decrease in the photosynthetic efficiency (Nyachiro et al., 2001; Chowdhury et al., 2021). For instance, Hede et al. (1998) evaluated the leaf chlorophyll content in (2 255) Mexican wheat landraces, which showed a high variability that positively correlated with grain weight. This was also reported by Reynolds et al. (1994), and Nyachiro et al. (2001) in spring wheat cultivars under heat and drought stress, respectively.

On the other hand, the use of portable chlorophyll metres for estimating chlorophyll is based on absorbance at two wavelengths; one measures light transmittance/absorbance, while the other the reflectance to determine the relative chlorophyll content. Now, chlorophyll metres are more available and affordable, such as atLEAF CHL, SPAD-502 Minolta, Opti-Sciences CCM-200, Apogee Instruments MC-100, METOS DUALEX, and PhotosynQ MultispeQ V1.0, that are well adapted to be used in field conditions (Zhu et al., 2012; Mendoza-Tafolla et al., 2019; Ali et al., 2020; Brown et al., 2022).

The efficiency of the portable chlorophyll metres has been compared with the laboratory (spectrophotometric) method, indicating a strong correlation among both methods. Additionally, equivalent performances have been observed between atLEAF CHL, a more affordable device, and SPAD values in various crops, including wheat, and in different environments (Zhu et al., 2012; Mendoza-Tafolla et al., 2019; Ali et al., 2020).

Hyperspectral analysis of chlorophyll combined with fluorescence imaging, offers higher sensitivity and spatial resolution compared to traditional methods alone. This approach allows continuous monitoring, to detect and manage crop stresses,

including drought. For example, in a study employing 335 wheat varieties, evaluating the chlorophyll content through hyperspectral imaging of flag leaves and the relationships with SPAD values at the grain-filling stage (under control and drought stress), Yang et al. (2023) demonstrated significant efficacy in detecting these stress responses in wheat.

The model in this study proved to be efficient for evaluating the chlorophyll content since it was strongly correlated with hyperspectral features in the visible region but weakly correlated in the near-infrared region. Moreover, there was a stronger correlation with SPAD values compared to reflectance in the spectral first derivative (Yang et al., 2023).

1.11 Exchange of resources from the plant to the AMF

1.11.1. Carbon (hexose sugars)

As part of the symbiosis, AMF increase the demand for carbon in the host plant. In exchange for mineral nutrients, the plant transfers between 4 to 25% of the photosynthesised carbon to the AMF (Parniske, 2008; Bonfante & Genre, 2010; Luginbuehl & Oldroyd, 2017). This carbon is typically transferred through the phloem in the form of sucrose from the plant shoot to the sugar delivery points in the roots. The plant utilises sucrose transporters (SUT), monosaccharide transporters (MST), and the recently identified SWEET (sugars will eventually be exported transporter) family, which includes importers and exporters of sucrose and sugars to protoplasts (Parihar et al., 2020).

However, AMF cannot utilise sucrose as a direct carbon source, as they lack the necessary enzymatic machinery to cleave it. Therefore, the plant host employs different enzymes, namely sucrose synthetase (SuSy) and invertase, to cleave sucrose into hexose sugars (i.e., monosaccharides or carbohydrates with six carbon atoms) (Garcia et al., 2016; Wang et al., 2017).

When sucrose is cleaved by SuSy, UDP-glucose (uracil-diphosphate glucose) and fructose are produced. Alternatively, if sucrose is hydrolysed by invertases, glucose and fructose are formed. These processes occur in the apoplast of the arbuscocyte, where hexoses are transferred to the intraradical hyphae using high-affinity MSTs by both the plants and the fungi (Figure 1.10).

For instance, in *Glomus* sp., two MSTs have been described: GpMST1 and RiMST2, which import glucose towards the intraradical hyphae (Parihar et al., 2020; Salmeron-Santiago et al., 2022). Interestingly, the expression of MSTs has been correlated with that of PHTs. Consequently, it has been proposed that the number of hexoses transported by the plant is proportional to the efficiency of PHTs by the fungus (Ji et al., 2022).

This correlation between hexoses and fungal PHTs is thought to be a mechanism to control or ensure that the AMF (through the extraradical hyphae), scavenge the soil, uptake and transport phosphate. Consequently, the plant reciprocally provides more hexoses to arbuscules connected with those resourceful zones; otherwise, the plant stimulates their premature senescence (Varma et al., 2017; Wang et al., 2017).

The transported hexoses are primarily incorporated into lipids, namely triacylglycerol (TAG), glycogen, and disaccharide trehalose. Eventually, through the glyoxylate cycle and/or gluconeogenesis, the TAG in the arbuscule is converted into carbohydrates and exported to the intra- and extraradical hyphae for utilisation in processes that require energy, such as the transport of mineral nutrients and the synthesis of proteins like glomalin. These are important processes that contribute to the functioning of the symbiosis (Bago et al., 1999; Luginbuehl & Oldroyd, 2017; Rich et al., 2017; Varma et al., 2017; Salmeron-Santiago et al., 2022).

1.11.2. Lipids

Lipids, which constitute more than 50% of the AMF biomass, are found in spores, vesicles (if produced), auxiliary cells, intraradical and extraradical hyphae, and arbuscules. However, AMF cannot synthesise lipids *de novo*; instead, it has been

established that they are transferred from the host plant (Olsson & Johansen, 2000; Hock, 2012; Souza, 2015).

The most common types of lipids in the AMF spores and vesicles are fatty acids (FA), which are the main components of phospholipids and neutral lipids such as TAG (with three FA esterified to glycerol), serving as energy and carbon stores. Palmitic acid (16:0) is the most frequently occurring FA, while palmitvaccenic acid (16:1ω5), although absent in plants, is found in AMF, some ectomycorrhizal fungi, and certain bacterial species. Additionally, 18:1ω7 and 20:1∂11 FA are described as AMF-specific, although they are not present in all species (Olsson & Johansen, 2000; Buchanan et al., 2015; Hock, 2012; Keymer et al., 2017).

Plants synthesise FA within plastids, using acetyl-coenzyme A (acetyl-CoA) as the substrate for the carbon backbone. Fatty acid biosynthesis initiates with an ATP-dependent carboxylation, catalysed by the enzyme acetyl-CoA carboxylase (ACCase), to form malonyl-CoA. Subsequently, malonyl-ACP is formed with an acyl-carrier protein (ACP) as a cofactor. Through a series of reactions, catalysed by fatty acid synthases (FAS), malonyl-ACP and acetyl-CoA assemble two carbons at a time to form long-chain FA with 16 or 18 carbons, such as TAG (Figure 1.10) (Buchanan et al., 2015; Bravo et al., 2017).

The term FAS is used to encompass all enzyme activities involved in FA biosynthesis, excluding ACCase. FAS can be divided into two types: type I FAS, usually found in eukaryotic cytoplasm and some bacteria, is characterised by multienzymes with various catalytic activities, either in a single polypeptide chain or as part of a large protein complex. Conversely, type II FAS is prevalent in plant organelles (such as chloroplasts and mitochondria) and in most bacteria. In this type, each enzyme relies on independent proteins for specific functions, and it also includes the ACP protein (Bravo et al., 2017; López-Lara & Geiger, 2018).

It has been demonstrated that common AMF species, such as *R. irregularis*, *Gigaspora margarita* and *Gi rosea*, lack genes encoding type I FAS, which are responsible for synthesising FA like palmitic acid. Conversely, mitochondrial type II

FAS genes, involved in synthesising octanoic acid (C8:0), are present. However, the biosynthesis of these FA only occurs inside colonised roots. This evidence supports the idea that AMF are FA auxotrophs, indicating that they rely on the host plant to receive lipids, which are then transported to the arbuscules. The exact mechanism of transport remains unknown (Trepaniér et al., 2005; Bravo et al., 2017; Jiang et al., 2017; Luginbuehl & Oldroyd, 2017b; Rich et al., 2017; Keymer & Gutjahr, 2018).

1.12 Quantification of AMF colonisation

Different approaches have been used for the identification of AMF, which can be classified as morphological (phenotypic characteristics), biochemical, and molecular (DNA or RNA sequences) (Table 1.7). However, biochemical approaches, which quantify sterols, chitin or FA are less commonly used methods since they lack taxonomic resolution (Alkan et al., 2004; Gamper et al., 2008; Voříšková et al., 2017).

Table 1.7 Overview of different approaches for the AMF identification, classified into morphological, biochemical, and molecular methods, highlighting the principles, advantages, and disadvantages of each method. Source: Morton & Benny (1990); Olsson et al. (1999); Redecker et al. (2003); Alkan et al. (2004); Smith & Read (2008); Öpik et al. (2009).

APPROACH	METHOD	PRINCIPLE	ADVANTAGES	DISADVANTAGES
MORPHOLOGICAL	Light microscopy	Visualisation of AMF structures in roots using staining techniques	Simple and widely used; allows direct observation of AMF structures and quantification	Cannot differentiate multiple species
	Spore morphology	Identification based on spore size, shape, and wall characteristics	Provides direct morphological data; useful for initial identification	Requires expertise in taxonomy; may not differentiate closely related species
BIOCHEMICAL	Fatty acid methyl ester (FAME) analysis	Profiling of fatty acid composition specific to AMF	Can provide specific biochemical markers for AMF	Requires specialised equipment and expertise; less commonly used
MOLECULAR	PCR with specific primers	Amplification of AMF-specific DNA sequences	High specificity and coverage of AMF groups (suitable for field samples)	Requires molecular biology expertise; potential for contamination
	q-PCR	Quantitative detection of AMF DNA in root samples	Highly specific and quantitative; can differentiate between AMF species	Requires specialised equipment and expertise
	Next-Generation Sequencing (NGS)	High-throughput sequencing of AMF DNA or RNA	Extensive identification of AMF communities; high resolution	Expensive; requires advanced bioinformatics skills

1.12.1. Morphological identification of AMF: spore diversity

Morphological identification and characterisation of AMF spores from different ecosystems have been used in studies of diversity and abundance. Although spore morphology is still used to some extent for identification of AMF species, this approach presents challenges. These challenges result from ambiguous morphological characteristics between AMF species, differences within the same species, and the spore development stage (Helgason et al., 2002; Lee et al., 2008; Krishnamoorthy et al., 2017).

Spores are the result of the asexual reproduction of the AMF at the sporulation stage. The sporulation process relies on many factors, such as the plant and fungus species, soil nutrient availability, temperature, and plant health, among other factors (Smith & Read, 2010). The spores are morphologically diverse; most are produced singly or in aggregations called sporocarps with a spherical, oval, oblong, or resembling a globose shape. They are formed from specialised hyphae either terminally, laterally, or intercalary inside the roots or outside in the soil (Walker et al., 2021).

Overall, spores exhibit thick walls that may be multi-layered that provide physical protection and resistance against desiccation. AMF spores are generally constituted by chitin, although some might contain β (1-3) glucan. Their size is widely variable; small spores range from 20 to 50 µm in diameter, while large spores can reach up to 500 µm in diameter (Brundrett et al., 1996; Smith & Read, 2010).

Internally, spores resemble cells because they contain organelles such as mitochondria, endoplasmic reticulum, vacuoles, and nuclei (Peterson et al., 2004). Spores also store lipids and some carbohydrates as reserves for germination, which may be microscopically identified as droplets or granules (Brundrett et al., 1996; Smith & Read, 2010).

Additionally, AMF spores are coenocytic or multinucleate, containing between 800 to 35,000 nuclei, but high numbers are also found in the hyphal tips, where cellular activity occurs for hyphal expansion. Nuclei can move bidirectionally or against the cytoplasmic flow, although the mechanism or force controlling their transit is unknown (Marleau et al., 2011; Taylor et al., 2015; Wipf et al., 2019; Kokkoris et al., 2020).

Nuclei are also exchanged during hyphal expansion, which could be a mechanism to conserve genetic diversity within spores (Giovannetti, 2008). Recently, it has been suggested that the genetic information in some AMF species can be organised either homokaryotically, with only one genotype present in their nuclei, or dikaryotically, with nuclei originating from two parental strains (Kokkoris et al., 2020).

Considering the large number of nuclei in the hyphal cytoplasm, it has been proposed that excess nuclei are degraded by a process called nucleophagy, a phenomenon observed in some fungi. Through selective autophagy, the fungus discards damaged DNA and is able to reuse the phosphorus and nitrogen. However, nucleophagy in AMF has not been directly studied (Kokkoris et al., 2020).

1.12.2. Morphological quantification of AMF: root staining

Root staining is one of the most used morphological quantification methods for calculating the percentage of AMF colonisation. Conventionally, this method involves clearing and staining the roots to observe fungal structures, such as (intraradical) hyphae and spores, arbuscules and vesicles, for their assessment (Ferrol & Lanfranco, 2020; Bodenhausen et al., 2021).

1.12.3. Fungal structures

During quantification of root colonisation, in general, two growing patterns can be identified, namely the *Arum* and *Paris* types, named after the plant genera in which they were first observed. In the *Arum*-type (Figure 1.11), the hyphae colonise the cortical cells intercellularly; then, they penetrate the cells to form the arbuscules (from the Latin *arbusculum*, small tree), a distinctive structure formed during the symbiosis to exchange resources between the plant and the fungus (Dickson, 2004; Taylor et al., 2015; Kobae, 2019).

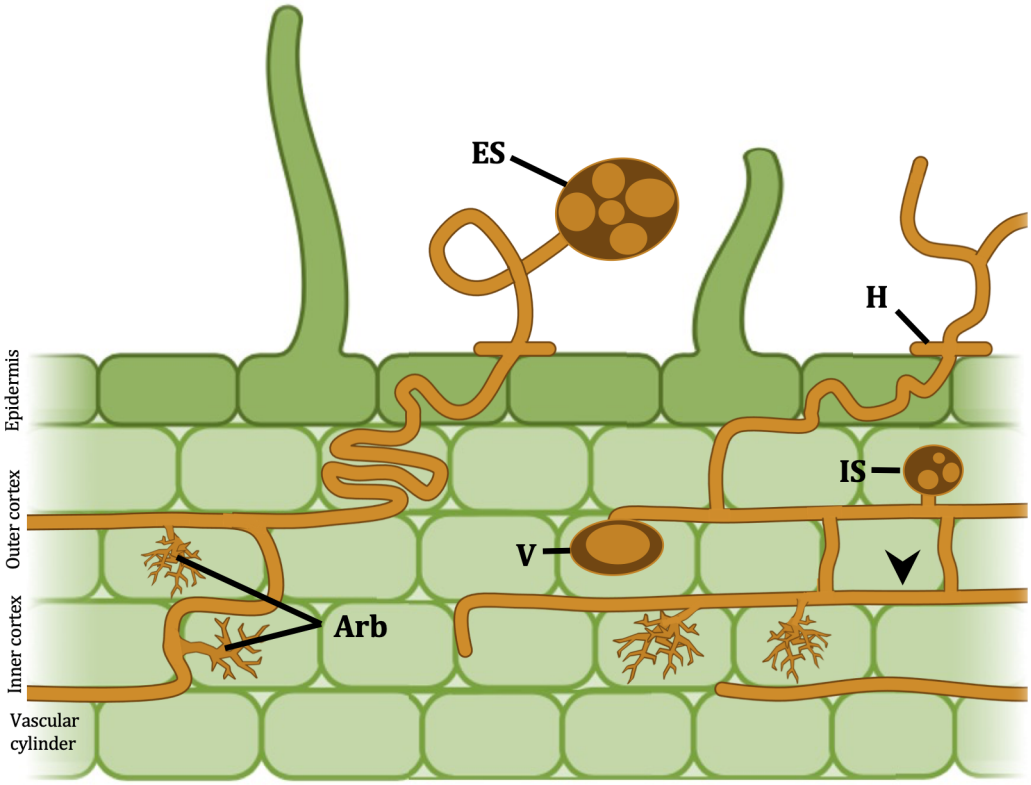


Figure 1.11 | Schematic representation of the *Arum*-type (intercellular) colonisation of cortical cells (arrowhead). An extraradical spore (ES) forms a fungal hypha that contacts the root epidermis; after the subsequent activation of the symbiosis-signalling pathway, the hypha flattens developing a structure called hyphopodium (H). Inside cortical cells, AMF form diverse structures, such as arbuscules (Arb), intraradical spores (IS) and vesicles (V) for functions like nutrient exchange, reproduction and storage. Based on Taylor et al., 2015.

Conversely, in the *Paris*-type (Figure 1.12) the hyphae follow an intracellular growth (from one cell to the adjacent) to the cortex, forming either hyphal coils, arbuscules, or both. Additionally, a combination of both morphological types of colonisations can also be found, called *Arum-Paris*-type continuum, in which the hyphae can move both intra and intercellularly (Redecker & Shüßler, 2014).

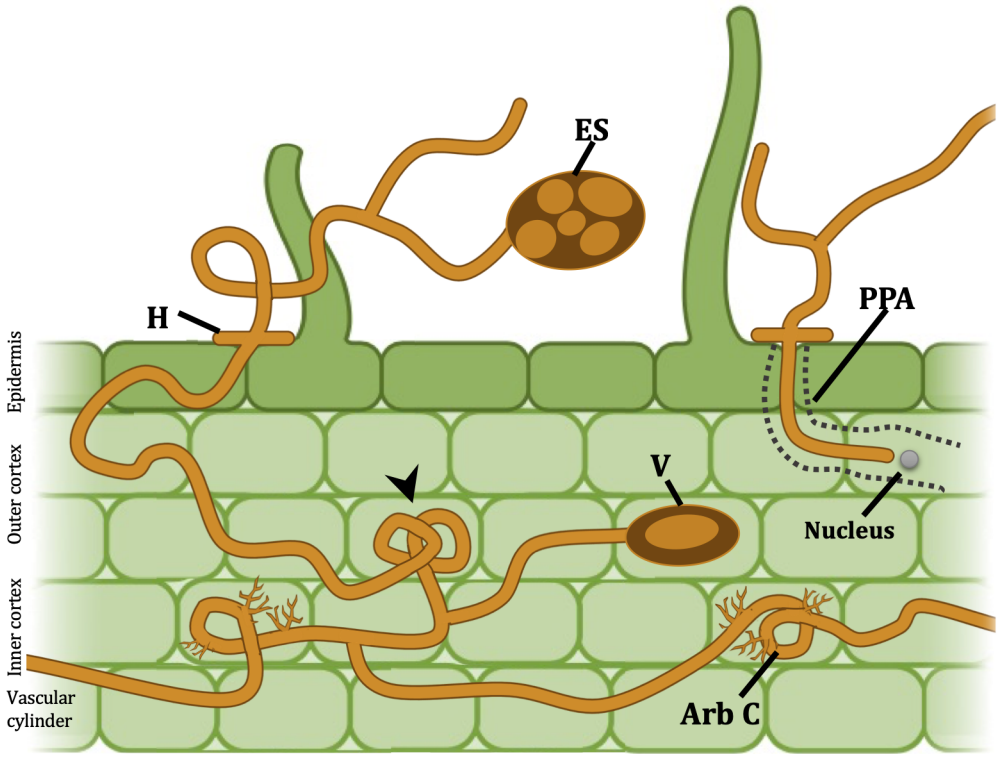


Figure 1.12 | Schematic representation of the *Paris*-type colonisation, in which the hyphae grow intracellularly in the plant root cortex. The formation of the prepenetration apparatus (PPA, dotted lines) starts after cellular modifications, where the hyphopodium (H) was formed. The hyphae led to the development of coiled arbuscules (Arb C), vesicles (V) and coil patterns (arrowhead). Based on Taylor et al., 2015.

Besides these colonisation patterns, AMF may display different capacities to form storage organs (vesicles) or spores either inside or outside the roots, and it has been suggested that factors such as the fungal and plant species or the combination of both, as well as the environmental conditions, may drive these factors (Dickson, 2004; Taylor et al., 2015).

1.12.4. Arbuscules

The AMF derive their name from the arbuscule, which is considered one of the most important aspects of the symbiosis because it is the site of nutrient interchange. In general, arbuscules comprise the trunk hypha and branched structures (Figure 1.13). Despite arbuscules are formed inside the cortical plant cells, they are not considered plant organelles because are separated from the plant cell cytoplasm by

the periarbuscular membrane (PAM) (Luginbuehl & Oldroyd, 2017; Pimprikar & Gutjahr, 2018; Kobae, 2019).

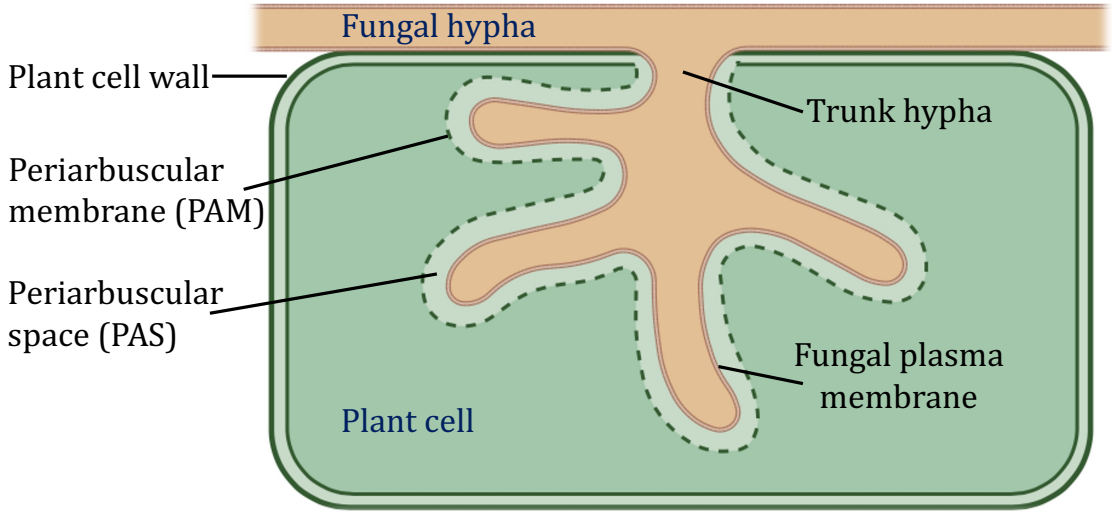


Figure 1.13 | Diagrammatic representation of an arbuscule. The arbuscule is surrounded by the periarbuscular membrane (PAM), which is derived from the plant, and separates the fungal plasma membrane from the plant cell cytoplasm. The space between the fungal plasma membrane and the PAM is known as the periarbuscular space (PAS), where the exchange of mineral nutrients occurs. Based on Lanfranco et al., 2018, created with BioRender.com (2023).

The area between the PAM and the fungal plasma membrane is the periarbuscular space (PAS), where the exchange of mineral nutrients occurs. In the PAS, phosphorus and nitrogen are the main mineral nutrients transported, delivered by extraradical hyphae from the soil (Bonfante & Genre, 2010; Gutjahr & Parniske, 2013; Luginbuehl & Oldroyd, 2017).

The development of the arbuscule can be divided into five stages (Figure 1.14). In Stage I, the plant nucleus is moved towards the hyphopodium and then to the opposite side of the cell, leading to the formation of the prepenetration apparatus (PPA). The PPA is a tunnel-like structure constituted of actin filaments, microtubules, Golgi bodies, and endoplasmic reticulum cisternae. Once this structure is created in the epidermal cell, the process is repeated in the adjacent cortical cells until it reaches the inner cortex (Genre et al., 2005; Luginbuehl & Oldroyd, 2017).

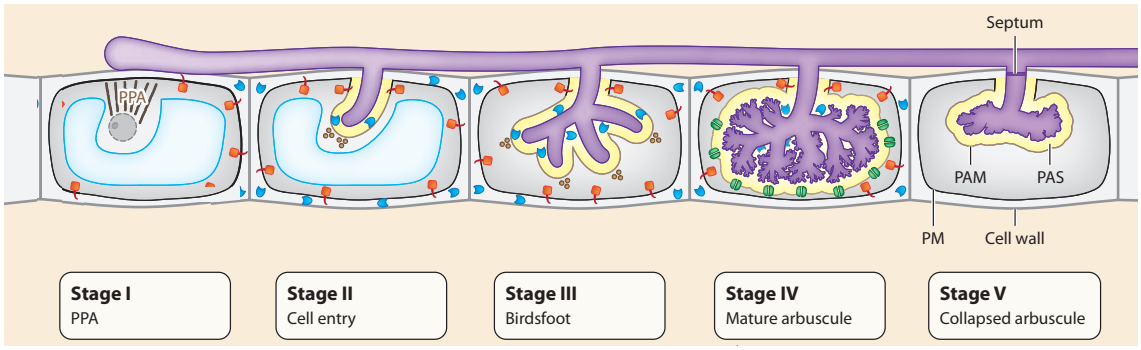


Figure 1.14 | Root colonisation and arbuscule formation by AMF. Stage I: the prepenetration apparatus (PPA) is formed; Stage II: following the PPA, the hypha penetrates the cortical cell and forms the trunk of the arbuscule; Stage III: formation of the primary arbuscular branching structures called birdsfoot; Stage IV: the arbuscule is mature, showing thin branching structures; and Stage V: collapsed arbuscule that, after a few days, decays, and a septum is formed to separate it from the hyphal network. Taken from Gutjahr & Parniske, 2013.

At Stage II, when the PPA is fully formed intracellularly, the new hypha can enter this tunnel-like structure. Once inside the inner cortex cell, the hypha can lead to the formation of the trunk hypha (the base of the arbuscule). Stage III is characterised by the formation of a primary branched structure, colloquially called birdsfoot, due to its resemblance in shape to a bird foot (Gutjahr & Parniske, 2013).

During Stage IV, the arbuscule continues developing until maturity, forming a fully thin-branched structure. The arbuscules have a short life, around 2 to 8 days; during that period, they will start to collapse with subsequent degeneration of the PAM and the related membrane proteins (Luginbuehl & Oldroyd, 2017; Pimprikar & Gutjahr, 2018).

In Stage V, after a few days, presumably after fulfilling its functions, the arbuscule collapses. This collapse is identified by the presence of a septum at the base of the trunk that disconnects the arbuscule from the intraradical and extraradical hyphal network. Thus, during the arbuscule assessment, it is very common to find the arbuscules at different developmental stages (Genre et al., 2005; Parniske 2008; Bonfante & Genre, 2010; Gutjahr & Parniske, 2013; Luginbuehl & Oldroyd, 2017).

The reasons for the relatively short life cycle of arbuscules are not yet fully understood, but it has been proposed that their lifespan is linked to the efficiency of mineral nutrient exchange of, which may be influenced by the host plant. Additionally, the plant can identify: (i) the most beneficial or efficient AMF species, i.e., the one that transports more mineral nutrients, and (ii) the arbuscules with extraradical hyphae connected to zones with available nutrients, which are more likely to proportionate resources (Parniske, 2008; Luginbuehl & Oldroyd, 2017).

Therefore, if arbuscules are linked to a depletion zone where mineral nutrients are low, arbuscules will be sanctioned by promoting premature senescence. Contrarily, if arbuscules efficiently transport more mineral nutrients, the host plant reciprocally will provide more hexoses (Parniske, 2008; Luginbuehl & Oldroyd, 2017).

Overall, the percentage of root colonisation by AMF provides a reliable approach for visualising and quantifying diverse intraradical fungal structures, as colonisation broadly mirrors various processes related with the growth and nutrition of plants (Alkan et al., 2004; Gamper et al., 2008; Voříšková et al., 2017). However, quantifying AMF root colonisation can be time-consuming, and observer-dependent, the latter may affect the interpretation of results. Additionally, this method may not detect very low levels or early stages of AMF colonisation, potentially underestimating the level of colonisation (Ferrol & Lanfranco, 2020; Bodenhausen et al., 2021).

1.12.5. Molecular quantification of AMF

Molecular approaches based on the polymerase chain reaction (PCR), have bridged the gap between spore morphological studies and genotypic analyses using fungal DNA and RNA. This approach was further enhanced by the design of specific primers that exclude the genetic material of the plant (Table 1.8), ultimately enabling the detection of diverse AMF species when combined with other techniques. Consequently, molecular markers have almost completely replaced morphological

and biochemical approaches (Alkan et al., 2004; Öpik et al., 2014; Taylor et al., 2017).

Table 1.8 Some primers designed for the AMF identification in diverse molecular approaches. Taken from Taylor et al., 2017.

PRIMER SET	TARGET REGION	NOTES	REFERENCE
NS1, NS2, NS4, NS5, NS8, ITS1, ITS4	SSU, ITS	Universal eukaryotic primers for nested PCR	White et al., (1990)
NS31	SSU	Universal eukaryotic primers	Simon et al., (1992)
AM1	SSU	Can amplify other groups of fungi, limited coverage of Paraglomeraceae	Helgason et al., (1998)
AML1, AML2	SSU	Longer fragment than NS31-AM1, improved AMF taxon coverage, amplifies some plants	Lee et al., (2008)
WANDA	SSU	Combined with NS31 10 bp toward 5' end from AM1, produces a shorter fragment for 454-pyrosequencing	Dumbrell et al., (2010)

Diverse molecular studies have focused on detecting conserved regions in different parts of ribosomal DNA (rDNA). The rDNA encodes ribosomal RNA (rRNA), which are involved in the production of ribosomes. In eukaryotes, it is commonly composed of repeated arrays of four or five genes. Although rDNA genes are highly conserved, they contain variable domains that can be used in phylogenetic studies for AMF identification (Gregory, 2005; Krishnamoorthy et al., 2017).

The rDNA unit is composed of two subunits (Figure 1.15). The first is the small subunit (SSU), which contains the 18S rRNA gene, and the second is the large subunit (LSU), which includes the 5S, 5.8S, and 28S rRNA genes. These subunits are separated by the internal transcribed spacers (ITS), which are variable regions among species. It has been suggested that the presence of conserved motifs in the ITS1 and ITS2 regions can be used for identifying fungal species (Gregory, 2005; Krishnamoorthy et al., 2017).

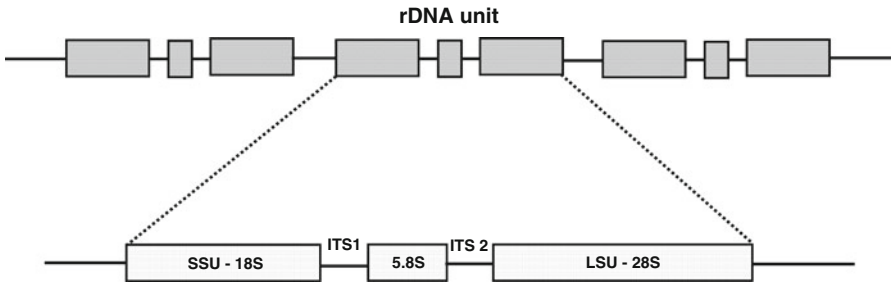


Figure 1.15 | Diagram of the ribosomal DNA (rDNA) unit and genes used for the arbuscular mycorrhizal fungi identification in the small subunit (SSU), large subunit (LSU) and the internal transcribed spacers (ITS). Taken from Krishnamoorthy et al., 2017.

The most targeted region in taxonomic studies has been the SSU rRNA gene, followed by the ITS and LSU rRNA genes. A two-marker system that combines both ITS and LSU regions results in a robust correlation with AMF species in database classifications (Schoch et al., 2012; Tedersoo et al., 2015). Complementing molecular approaches with morphological ones could provide a better understanding of the AMF colonising plant roots compared to the AMF spore community in the soil (Redecker et al., 1997; Krüger et al., 2009; Öpik et al., 2014; Taylor et al., 2017).

1.12.6. Molecular quantification of root colonisation

Quantification of AMF-colonised plant roots using quantitative real-time PCR (qPCR) is a suitable technique for determining AMF abundance or for relative quantitative evaluations. However, qPCR abundance estimations targeting rRNA genes can fluctuate due to variability in this region among different taxa. Therefore, reliable qPCR quantification of AMF in roots can be achieved by comparing or calibrating the detected signal with results obtained using conventional microscopy methods (Taylor et al., 2017; Voříšková et al., 2017; Bodenhausen et al., 2021).

Results obtained from genetic quantification methods, such as qPCR, should be interpreted with caution since different factors may influence the findings. For instance, the presence of multiple nuclei contained in hyphae or spores of a specific AMF species, or during co-colonisation events, may increase the content of nucleic acids. This increase can potentially lead to higher signals detected during genetic

quantification. Therefore, in some cases, results from genetic quantification should be considered indicative rather than definitive (Ferrol & Lanfranco, 2020; Janoušková & Caklová, 2020).

1.12.7. High-throughput sequencing

High-throughput sequencing, also known as next-generation sequencing, refers to different technologies that can sequence multiple samples simultaneously with high throughput, scalability and speed. One of the advantages of these technologies is that they can analyse low DNA concentrations of samples of microbial communities to generate relative abundances of the microorganisms. Several high-throughput platforms have been developed over years, including the SOLiD system (Life Technologies), the 454-sequencing system (Roche) and more recently the MiSeq, HiSeq and NovaSeq platforms (Illumina), each offering unique features and capabilities (Gorzelak et al., 2012; Asemaninejad et al., 2016; Piro, 2020).

Among these technologies, the Illumina MiSeq platform has become the most widely used in studies involving fungi and other microorganisms, since it was introduced as a compact sequencer using the *sequencing by synthesis* technology of Illumina. This approach can be divided into three major steps (Figure 1.16).

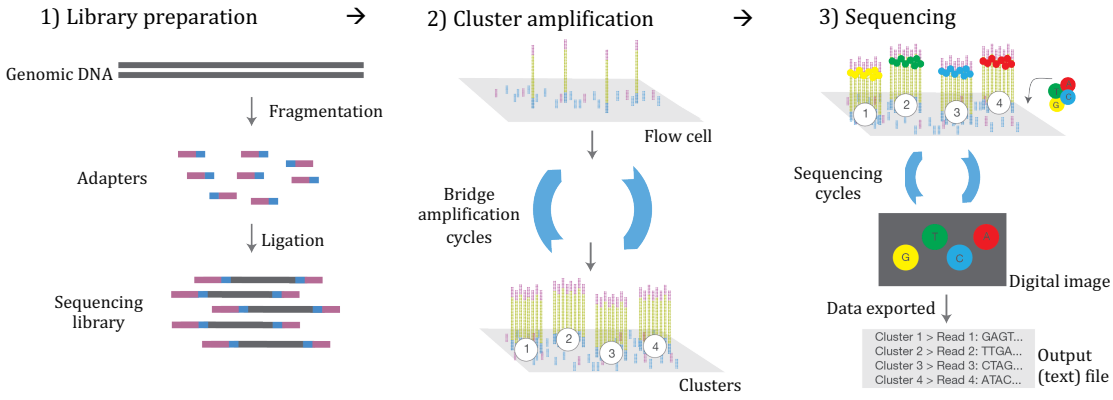


Figure 1.16 | Overview of the next-generation sequencing process: 1) Library preparation. The adapters are annealed to the extremes of the DNA fragments. 2) Cluster amplification. The DNA fragments bind to adapters on the flow cell, and through the bridge amplification they produce

clusters of DNA fragments. 3) Sequencing. During each sequencing cycle, one fluorescently labelled deoxynucleoside triphosphate (dNTP) is added to the growing DNA strands, a laser excites the fluorophores in all the fragments that are being sequenced. Subsequently, the fluorescence is detected by a camera, and a computer identifies the nucleotide. Then the sequencing terminator is removed, and the next sequencing cycle starts. Modified from www.illumina.com/technology/next-generation-sequencing.html, and Young & Gillung, 2020.

1) Library preparation: In most next-generation sequencing applications, DNA fragments are typically ligated to small adapter sequences of DNA, followed by attachment to either the forward or reverse primer. Additionally, two different oligonucleotides immobilise, or anchor, these adapter sequences to the flow cell. The flow cell, typically a glass slide (depending on the Illumina platform), contains one or more channels or lanes where these synthesised sequencing adapters allow DNA fragments to bind (www.illumina.com; Schirmer et al., 2015; Ravi et al. 2018; Young & Gillung, 2019).

2) Cluster amplification: After DNA fragments with ligated adapters are added to the flow cell coated with oligonucleotides, a complementary DNA strand is synthesised. Subsequently, the double-stranded DNA fragment is denatured, and the original DNA template along with unbound fragments are washed off the flow cell. Following this, a process called bridge amplification is carried out: the immobilised single-stranded DNA fragment bends, and the adapter at the other end binds to the nearest complementary oligonucleotide on the flow cell (www.illumina.com).

Therefore, the bent single-stranded DNA fragment is amplified by action of the DNA polymerase, forming a double-stranded bridge. This process is repeated simultaneously for millions of clusters, resulting in the clonal amplification of the original DNA fragments. Once completed, all the reverse strands are cleaved and washed off, resulting in hundreds of millions of forward single-stranded DNA fragments clusters (www.illumina.com; Schirmer et al., 2015; Ravi et al. 2018; Young & Gillung, 2019; Piro, 2020).

3) Sequencing: The bridge amplification starts with the addition of a primer and a fluorescently labelled deoxynucleoside triphosphate (dNTP, i.e., A, C, T and G), which

are incorporated into the nucleic acid chain and serve as terminators of the synthesis. After the incorporation of the first dNTP, the DNA polymerase and unbound dNTPs are washed off. Subsequently, the flow cell is illuminated with lasers to activate the fluorescent tag of the recently incorporated nucleotide (each nucleotide gives a different colour) (www.illumina.com; Schirmer et al., 2015; Ravi et al. 2018; Young & Gillung, 2019; Piro, 2020).

The fluorescence is then detected by a camera, and a computer identifies the nucleotide. This illumination process also leads to the cleavage of the fluorescent tag. This cycle is repeated until the DNA fragments are completely amplified. Finally, the sequencing data is exported to a file for further analysis (www.illumina.com; Schirmer et al., 2015; Ravi et al. 2018; Young & Gillung, 2019; Piro, 2020).

The accessibility and relatively lower costs of next-generation sequencing technologies have led to a better knowledge of AMF diversity and distribution. In molecular-genetic studies of AMF, partial sequences are amplified from DNA samples (obtained from single spores, colonised roots, soil, etc.) using PCR with specific primers. The selection of these primers is critical, and ribosomal DNA genes from the SSU, LSU, and ITS regions are commonly selected for amplification to obtain a reliable community composition (Suzuki et al., 2020).

However, there are some considerations with AMF, due to their high genetic variation, including the SSU–ITS1–5.8S–ITS2–LSU region. Therefore, the choice of region used for molecular-genetic identification presents both advantages and disadvantages (Victorino et al., 2021). For instance, the SSU gene, a highly conserved region, is commonly chosen since primer pairs can efficiently classify it into virtual taxa in various databases. Additionally, SSU gene primers efficiently recognise the majority of AMF families, although they may not adequately classify some AMF species (Schoch et al., 2012; Tedersoo et al., 2015; Kryukov et al., 2020; Victorino et al., 2020).

The ITS region is routinely used to identify general fungi; however, primers targeting this region are also available for AMF community studies. The ITS1 region

is considered highly variable, even within individuals, making it difficult to discriminate among AMF species. In Illumina MiSeq analyses, it is recommended to use the ITS2 or the full ITS region, which provides a reliable AMF identification (Schoch et al., 2012; Tedersoo et al., 2015; Kryukov et al., 2020; Victorino et al., 2020).

1.13 Thesis aims and hypotheses

Since wheat landraces have the ability to form beneficial symbiotic relationships with AMF and vice versa; root colonisation (of AMF) could enhance diverse morphological plant traits, which is valuable for adaptation to unfavourable environmental conditions, particularly drought stress, in light of recent climate changes (Gharib et al., 2021).

It has been studied that tolerance or adaptation to drought stress in wheat is through variation in phenological traits, such as plant height, the number of tillers and leaves per plant, root and shoot dry weights, flag leaf area, and overall leaf area per plant, among others (Bahadur et al., 2019). However, most studies have focused on commercial wheat varieties or cultivars to study or develop their characteristics, resulting in very limited knowledge about the drought tolerance of wheat landraces.

Consequently, this study conducted a greenhouse trial to evaluate morphological and physiological parameters that could serve as indicators to answer to the following question: How do AMF influence various morphological parameters in wheat landraces to help them cope with drought stress?

Moreover, it has been suggested that inoculation with AMF significantly enhances drought stress tolerance in colonised wheat plants compared to those under well-watered conditions. Furthermore, root colonisation by AMF has been shown to improve grain production in wheat plants under drought stress (Al-Karaki et al., 2003). Thus, another related question arises: Does AMF inoculation increase wheat landraces growth, and grain yield under drought stress?

These questions were addressed using a panel of spring wheat landraces, referred to as the YoGI panel (Barratt et al., 2023), which were selected from multiple germplasm collections representing a variety of wheat-growing regions and environmental conditions. By using landraces from diverse regions (MEs), the experiment aims to identify inherent characteristics and adaptive mechanisms that

contribute to drought tolerance. We hypothesised the following: (i) Drought stress would negatively affect the development and production in four selected wheat landraces, and (ii) inoculation with AMF would ameliorate these negative effects by diverse mechanisms, reflected on the increase of various plant parameters. (iii) Colonisation with AMF would significantly increase wheat productivity.

This approach will expand our understanding of how these wheat landraces perform under drought stress in greenhouse conditions. Besides, the insights obtained from this research could be applied to the selection of other wheat landraces from the YoGI panel or to wheat-breeding programmes aimed at developing drought-tolerant varieties, using both morphological parameters and molecular approaches.

The specific objectives of this thesis are outlined below, corresponding to the different chapters:

1.13.1. Aims Chapter 2:

1) To assess the effects of drought stress on four spring wheat landraces inoculated with AMF (soil inoculum) across different morphological and physiological parameters:

- Total chlorophyll content at vegetative and reproductive stages
- Dry biomass (roots and aerial part i.e., shoots and leaves)
- Plant height
- Grain yield

2) To calculate the percentage of root colonisation by AMF in four spring wheat landraces and analyse its relationship with drought stress.

1.13.2. Aims Chapter 3:

1) To evaluate the fungal community composition in four spring wheat landraces under two irrigation levels using two sets of primers: ITS for

identifying general fungi, and WANDA-AML2 for AMF, through next-generation sequencing (Illumina MiSeq).

2) To compare the fungal community composition within wheat root landraces based on both sequencing results.

3) To investigate whether fungal communities or their abundance are associated with plant morphological and physiological parameters.

1.13.3. Aims Chapter 4:

1) To estimate the effects of drought stress on 30 spring wheat landraces inoculated with a single AMF species, *Rhizophagus irregularis* BEG72, using various morphological parameters:

- Total chlorophyll content at vegetative and reproductive stages
- Dry biomass (roots, aerial part, and inflorescence)
- Inflorescence (length)
- Plant height
- Grain yield
- Grain number

2) To determine the percentage of root colonisation by AMF using relative quantification (qPCR), and to compare it with the percentage of colonisation obtained by microscopy.

Chapter 2. Effect of drought stress on root colonisation by AMF in four spring wheat landraces

2.1 Introduction

Numerous studies have demonstrated the positive benefits of symbiosis between wheat plants and AMF, including enhanced plant nutrition, mineral cycling, and overall growth and development (Chahal et al., 2020). These advantages extend beyond wheat, as AMF symbiosis has also been shown to improve growth and productivity in various other crops, such as maize, rice, tomato, and potato. However, crop production is increasingly threatened by drought stress, intensified by climate change. Drought can occur at any stage of plant development, depending on local environmental conditions, and it negatively impacts almost all aspects of plant growth (Lu et al., 2015; Sabia et al., 2015; Hart et al., 2015).

Drought tolerance in plants, particularly in staple crops like wheat, is a complex phenomenon, involving a variety of physiological, morphological, and molecular traits. Identifying and characterising these traits have become critical in plant breeding programmes, as they can accelerate the development of drought-tolerant wheat varieties (Farooq et al., 2014; Budak et al., 2015; Mwadzingeni et al., 2016).

Moreover, drought conditions can significantly alter soil microbial communities, including AMF and other beneficial microorganisms. This is particularly concerning because AMF play a vital role in enhancing plant tolerance to drought and influencing crop performance (Anjum et al., 2011; Miransari, 2014). For example, Porcel & Ruiz-Lozano (2004) found that AMF colonisation increases plant resilience to drought through several mechanisms, such as enhanced water uptake via extensive hyphal networks, improved nutrient acquisition (especially phosphorus), and modifications to plant physiology and biochemistry. Although AMF colonisation rates declined under drought, the remaining colonisation still provided substantial benefits to the host plant.

In a field experiment, Duan et al. (2021) demonstrated that AMF inoculation under non-irrigated conditions significantly increased various parameters in wheat, including aboveground biomass, crop productivity, water-use efficiency, and rhizosphere soil organic carbon, compared to non-inoculated treatments.

Thus, by enhancing physiological, morphological, and molecular traits, AMF colonised wheat plants can exhibit improved drought tolerance, which is crucial for maintaining productivity in water-limited environments. Optimising AMF colonisation offers a promising strategy for developing drought-resistant wheat cultivars (Augé, 2001; Zou et al., 2019).

2.1.1. Aims and objectives

The first objective of this study was to evaluate the impact of drought stress on two key areas: (a) the root colonisation by AMF in four distinct spring wheat landraces (designated as A5, A7, B5, and C3), and (b) various plant variables, which are categorised as follows: (i) physiological parameters, including chlorophyll content measured at both vegetative and flowering stages, (ii) morphological parameters, encompassing aerial and root dry biomass, and plant height, and (iii) grain yield components, specifically focusing on grain weight and the number of grains per plant.

Here, it was hypothesised that drought stress will differentially impact the morphological and physiological parameters of the four spring wheat landraces inoculated with AMF, with the expectation that AMF inoculation will mitigate some of the adverse effects of drought, specifically, enhancing total chlorophyll content, increasing or maintaining dry biomass (both roots and aerial parts), maintaining plant height, and improving grain yield, compared with a non-inoculated control treatment.

The second objective was to calculate the percentage of root colonisation by AMF in the four spring wheat landraces. Although the effect of drought stress on root colonisation by AMF can vary among different wheat landraces, reflecting genetic

diversity in response to environmental stresses. It is hypothesised that AMF root colonisation will be correlated with better drought stress resistance, mirrored on the morphological and physiological plant performance.

Thus, by understanding and exploiting morphological and physiological traits such as chlorophyll content, dry biomass accumulation, plant height, grain yield, and adaptation to unfavourable conditions, serve as valuable indicators in the screening and selection of wheat landraces to be hybridised with commercial wheat varieties to develop new genotypes with improved characteristics. This approach influences the genetic diversity and adaptability of landraces, contributing to the development of high-performing wheat varieties (Gianinazzi et al., 2010; Zhan et al., 2020).

2.2 Materials and methods

2.2.1. Plant material: wheat diversity panel

A collection of *T. aestivum* landraces were used, the YoGI biodiversity panel (Barratt et al., 2023) was derived from project (**Yo**rk) BBSRC **G**CRF **I**AA: Functional genotypes for worldwide wheat diversity panel (Harper Lab). The panel is composed of different germplasm compendiums: Watkins Landrace Collection (John Innes Centre) in England, landraces from CIMMYT in the United Mexican States, and the Research Institute of Crop Production (RICP) in Czechia. These are derived from *c.*60 countries representing all the different wheat MEs.

For this experiment, four spring wheat landraces from the YoGI panel were used, from distinct MEs to represent specific agro-ecological zones with unique challenges, particularly regarding water availability. Table 2.1 shows the assigned names of the four genotypes and the origin area.

Table 2.1 Spring wheat landraces from the YoGI collection used in this experiment. The assigned name, area of origin, and mega-environments (MEs) are also indicated.

LANDRACE		NAME	ORIGIN	MEs
1.	YoGI_205	**A5**	Afghanistan	7
2.	YoGI_207	**A7**	Afghanistan	7
3.	YoGI_005	**B5**	Bolivia	3
4.	YoGI_333	**C3**	Colombia	1

Wheat landraces A5 and A7 were collected from different areas of Afghanistan, which present a ME7, characterised as an optimum environment for wheat production, where conditions are generally favourable for growth due to the availability of irrigation, minimising the risk of drought stress and allowing for the cultivation of high-yielding wheat varieties. Landrace B5 with a ME3 is delineated by high rainfall and the presence of acidic soils, creating challenging conditions for wheat cultivation, as it often results in nutrient imbalances, such as aluminium toxicity and deficiencies in nutrients like phosphorus (Rajaram & vanGinkel., 2001; Reynolds et al., 2007; Reynolds, 2010).

Lastly, landrace C3 from ME1 shows irrigated, temperate conditions that provide a stable climate favourable to wheat growth. The availability of irrigation ensures that water supply is consistent throughout the growing season, minimising the risk of drought stress (Rajaram & vanGinkel., 2001; Reynolds et al., 2007; Reynolds, 2010).

2.2.2. Plant growth conditions

Wheat seeds were washed in a solution (30% sodium hypochlorite with 0.02% Triton X-100) for 15 minutes, followed by three rinses with distilled water. Subsequently, the seeds were placed on water-soaked tissue paper in square Petri dishes, which were then covered with aluminium foil and kept at room temperature for four days to stimulate seed germination. Based on prior standardisation of the germination process, it was determined that seed stratification was not required.

Two germinated seedlings for each of the four genotypes per pot were sown in 1.5 L pots (17 cm in diameter and 13 cm in height), with three replicates per treatment (n=6). The pots were filled with a substrate consisting of sand:Terra-Green®:soil mixture at a ratio of 45:45:10 (v/v/v). Terra-Green® (Oil-Dri, Cambridgeshire, UK) is a calcined attapulgite clay soil conditioner.

All pots were kept in a greenhouse under temperature conditions ranging from 12.8 to 30°C, with 10-14 h of ambient sunlight and 14-10 h of ambient darkness. The pots were arranged in a randomised block design to prevent environmental gradients that might influence plant development. The wheat plants were fertilised with modified Hoagland nutrient solution (low in N and P, as in Scheublin & van der Heijden, 2006), containing 6 mM KNO3, 0.5 mM Ca(NO3)2, 3.5 mM CaCl2, 1 mM NH4H2PO4, 1 mM MgSO4, 50 µM KCl, 25 µM H3BO3, 2 µM MnSO4, 2 µM ZnSO4, 0.5 µM CuSO4, 0.5 µM (NH4)6Mo7O24, 20 µM Fe(Na)EDTA (Hoagland & Arnon, 1950). The plants were grown in the greenhouse (April 2019 to June 2019) until harvest, approximately 42 days after sowing.

2.2.3. Inoculation and irrigation treatments

Six pots (12 plants) were inoculated using soil as live AMF inoculum. The soil samples were taken in March 2019 from a wheat field at the University of Leeds Research Farm (Wise Warren Farm, Warren Lane, Tadcaster, England). The farm is located between Leeds and York, UK covering approximately 317 hectares, bringing together four farmsteads. Approximately 75% of the land is used for arable farming, including wheat, barley and oil seed rape. Soils are typically shallow, well-drained, calcareous, and fine loamy soils (Aberford Series). The annual average rainfall is 674mm, with August being the wettest month (65.9mm) and February the driest (46mm). Average temperatures range from a low of 4°C in January to a high of 16°C in August, with an annual average temperature of 9°C (www.leeds.ac.uk/global-food-environment-institute/doc/gfei-smart-farm).

The live AMF inoculum (soil) was analysed to confirm the presence of AMF propagules. Wheat root samples were stained and examined under a microscope to quantify AMF colonisation based on the presence of fungal structures. Additionally, soil samples were analysed to isolate spores for observing morphological diversity, though species identification was not conducted.

Control plants were given sterilised inoculum (soil), which was autoclaved twice, two days apart, to allow any microorganisms to grow before being sterilised again. The sterilised inoculum, comprising 10% of the total pot substrate volume, was then added to the pots. Additionally, both inoculated and control plants received 5 mL of a filtered soil suspension (sieved to exclude AMF spores >32 μm) to ensure a similar rhizobacterial population across treatments.

In this research project, two irrigation treatments were implemented based on field capacity (FC) of the soil, which was estimated using the following equation: FC = 100 (W2 − W1)/W1, where W1 represents the dry weight of pot substrate. The pots were then irrigated until saturation, then covered to prevent water evaporation from the surface. After a 48-hour rest period, the pots were weighed again to obtain W2, the weight at saturation. The difference between W2 and W1 indicates the

amount of water retained by the substrate, allowing calculation of the FC percentage (Ould Amer et al., 2023).

Well-watered plants were maintained at 95% FC, while drought-stressed plants were kept at 40% FC as in Abid et al. (2016); Cui et al. (2017); Martínez-Goñi et al. (2023); Ishfaq et al. (2024). Drought-stressed plants were irrigated by daily weighing of the pots, with water loss due to evapotranspiration being replaced to maintain their original weight. The drought stress treatment was applied from the second week after planting and continued until the conclusion of the plant life cycle.

2.2.4. Leaf chlorophyll measurement

Total leaf chlorophyll content was measured weekly during the vegetative growth stage (week 1) through to the onset of anthesis and flowering (week 8) using a chlorophyll fluorescence metre, atLEAF CHL BLUE (FT Green LLC, Wilmington, DE) (Zhu et al., 2012). The device estimates chlorophyll content by inserting a leaf into the measuring window, producing values ranging from 0 to 99.9 (atLEAF CHL value). These atLEAF CHL values were then converted to total chlorophyll content, expressed in milligrams per square centimetre (mg/cm^2), using the conversion tool provided on the website of the manufacturer (https://www.atleaf.com).

2.2.5. Plant dry biomass production

The aerial parts of the plants, including shoots and leaves, were harvested at the end of the experiment and dried in an oven at 70 °C for 48 to 96 hours, after which their dry weight was recorded. The root systems of each plant were carefully removed from the pots, manually washed to remove any attached substrate, and then rinsed with deionised water. The cleaned roots were frozen at -80 °C overnight and subsequently freeze-dried using a Scanvac freeze dryer (LaboGene, Lynge, Denmark), after which their dry weight was recorded (Janoušková et al., 2015). Additionally, at the conclusion of the experiment, plant height was measured from the base of the shoot to the tip of the spike. At plant maturity, grain yield and grain number were also recorded for each wheat landrace.

2.2.6. Percentage of AMF root colonisation

A dry root sample from each wheat plant was taken and cut into 1 cm lengths for microscopic examination to assess the presence of AMF structures. The root samples were prepared for staining by first rehydrating them in tap water overnight. Following rehydration, the roots were cleared to remove any pigments and cellular contents that might obscure the visibility of fungal structures by adding a 10% potassium hydroxide (KOH) w/v solution in a 2 mL plastic vial. The vials were then incubated in a water bath at 70 °C for 45 minutes and subsequently rinsed three times with tap water.

Next, the cleared roots were acidified with a 5% hydrochloric acid (HCl) v/v solution and stained using the trypan blue method (Phillips & Hayman, 1970). The stained root segments were then aligned in rows parallel to the long axis of a slide and observed at 200x magnification under a microscope (Carl Zeiss, West Germany) to determine the percentage of colonisation using the magnified intersections method (McGonigle et al., 1990).

For each plant replicate, a total of 100 passes were examined across the root segments. AMF structures intersected by the vertical crosshair in the eyepiece such as arbuscules, hyphae, spores, or vesicles were recorded (Figure 2.1). The total percentage of colonisation, as well as the percentage for each fungal structure, was then calculated. If no fungal or non-AMF structures were observed in the root, it was marked as negative.

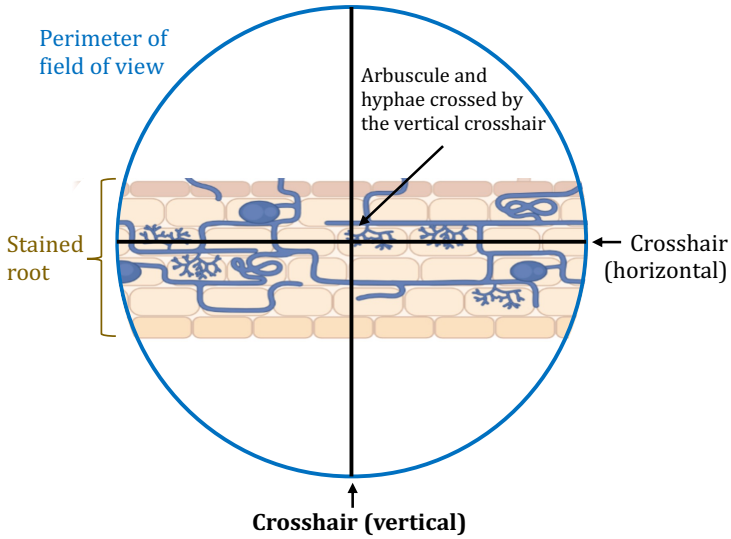


Figure 2.1 | Diagram showing how the magnified intersections method (McGonigle et al., 1990) is applied. Stained roots mounted in a glass slide are observed at 200x magnification in a microscope with a crosshair in the eyepiece. When the vertical crosshair crosses one or more structures, the appropriate category increments by one in a total of 100 passes. Intersections were classified and counted as follows: arbuscules, hyphae, intraradical spores, vesicles or negative (blank root segment or non-AMF structures). Adapted from BioRender.com (2020).

2.2.7. DNA extraction and PCR amplification

Total genomic DNA was extracted from freeze-dried root samples of each pot (two wheat plants) for all treatments using the DNeasy® Plant Extraction Kit (Qiagen, Valencia, California), following the protocol provided by the manufacturer. A first PCR that identifies the internal transcribed spacer region (ITS) was carried out to observe the presence of general fungi in the samples. Additionally, for the identification of the arbuscular mycorrhizal fungi, two PCRs that amplify the small subunit ribosomal RNA (SSU rRNA) gene using specific primers (Table 2.2) were accomplished.

Table 2.2 Primer pairs used in this experiment. (F), Forward primer; (R), Reverse primer. SSU, small subunit rRNA gene; ITS, Internal Transcribed Spacer.

PRIMER PAIR	SEQUENCE (5' → 3')	SIZE (BP)	TARGET REGION	REFERENCE
ITS1 (F)	TCC GTA GGT GAA CCT GCG G	580-750	ITS	White et al., 1990
ITS4 (R)	TCC TCC GCT TAT TGA TAT GC			

AML1 (F)	ATC AAC TTT CGA TGG TAG GAT AGA	800	SSU	Lee et al., 2008
AML2 (R)	GAA CCC AAA CAC TTT GGT TTC C			
WANDA (F)	CAG CCG CGG TAA TTC CAG CT	550-600		Dumbrell et al., 2011

The PCR amplification of both the ITS and the SSU rRNA, were performed using the following components: 0.1 mM dNTPs, 10 pmol of each primer, 5 U of Taq DNA polymerase and the reaction buffer (Promega) with a total volume of 20 µL. The PCR program to detect the ITS was as follows: initial denaturation at 94 °C for 3 minutes, followed by 30 cycles at 94 °C for 30 seconds, 55 °C for 1 minute, and 72 °C for 1 minute, finally, an extension period of 72 °C for 10 minutes.

As mentioned above, primers AML1-AML2 were used to identify the SSU rRNA gene, with the PCR program as follows: initial denaturation at 95 °C for 2 minutes, followed by 30 cycles at 94 °C for 30 seconds, 59 °C for 1 minute, and 72 °C for 1 minute, finally an extension period of 72 °C for 10 minutes. The resulting amplicons were utilised to do a semi-nested PCR using a 1:10 or 1:100 dilution, which was carried out using the aforementioned PCR components and program with the primers WANDA-AML2. The presence of the corresponding amplicons (with the expected size) was confirmed using agarose gel electrophoresis.

2.3 Statistical analyses

Data are presented as mean ± SE (standard error) of six independent biological replicates. Data were subjected to a three-way analysis of variance (ANOVA, $p \leq 0.05$) to evaluate the effects of the landrace (A5, A7, B5, and C3), inoculation (AMF-inoculated and non-inoculated control), irrigation (drought stress and well-watered) treatments, as well as their interactive effects on chlorophyll content, percentage of root colonisation (AMF and non-AMF), aerial dry biomass, root dry biomass, plant height, grain yield, and grain number. Followed the honest significant difference test by Tukey, using a significance level of 5% ($p \leq 0.05$). Normality of residuals was tested using the Shapiro-Wilk test.

Finally, to evaluate the relationship between two variables, a Pearson correlation coefficient analysis was conducted. The correlation coefficient (R) was used to describe the nature of the relationship: no correlation (0), positive linear correlation (+1), or negative linear correlation (-1), along with the significance value (p). All statistical analyses were performed using RStudio software (v. 4.2.2).

2.4 Results

2.4.1. Changes in chlorophyll levels during drought stress

Chlorophyll content is a critical indicator of plant health, closely linked to nitrogen availability and overall photosynthetic efficiency (Bojović & Marković, 2009). In this research project, total chlorophyll levels (chlorophyll *a* and *b*) were measured in four spring wheat landraces, both inoculated with AMF and non-inoculated, under drought and well-watered conditions.

During the vegetative stage, chlorophyll content was measured from week 1 (Zadoks scale, Z10) to week 5 (Z41). The analysis of variance (ANOVA) showed no significant differences in chlorophyll content between the treatments, regardless of the inoculation status or the irrigation conditions (p >0.05). The chlorophyll content at the flowering stage (Figure 2.2), measured from the anthesis (Z43) to the beginning of senescence (Z79), revealed a significant reduction in chlorophyll levels due to drought stress (p <0.001), regardless of AMF inoculation.

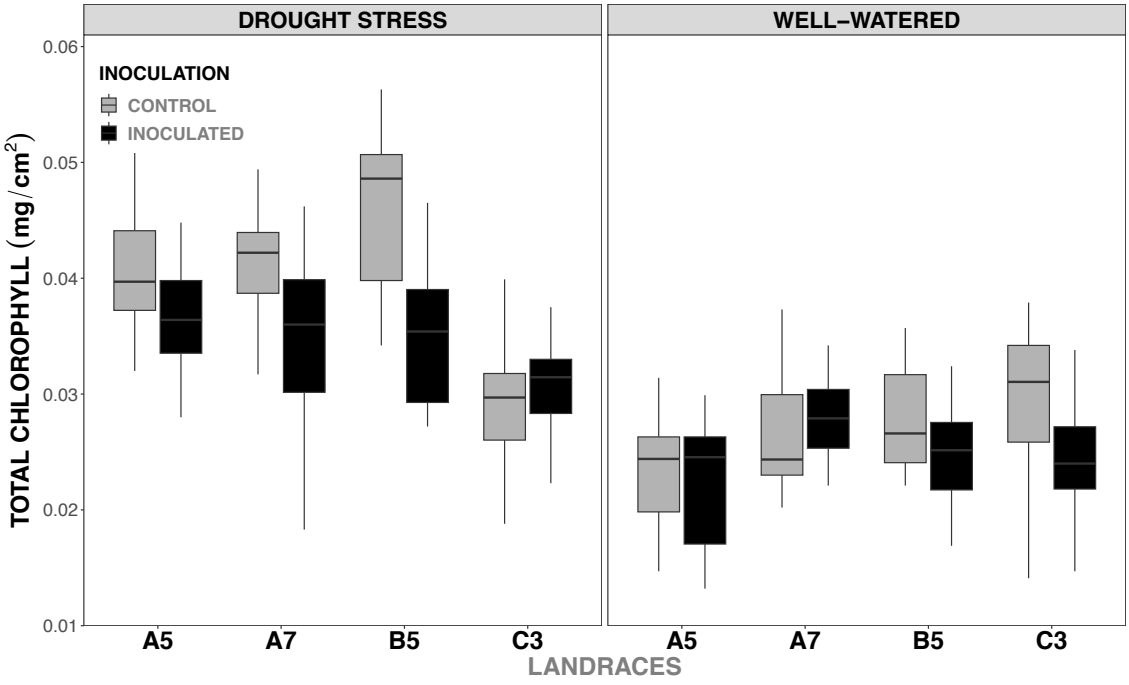


Figure 2.2 | Total chlorophyll content (mg/cm^2) during the flowering stage across four different spring wheat landraces (A5, A7, B5, and C3) under two different irrigation conditions: drought stress (left panel) and well-watered (right panel), and two inoculation treatments: non-inoculated (control, grey bars) and inoculated with AMF (black bars). Thick lines represent medians, boxes indicate interquartile ranges, and whiskers show minimum and maximum chlorophyll values. Means with the same letter are not significantly different at the 0.05 level using Tukey's HSD test (n= 6).

The data from this parameter revealed an unexpected trend: all wheat plants subjected to drought stress exhibited a significant increase in chlorophyll levels, regardless of the inoculation status, compared to those grown under well-watered conditions.

Under drought stress, wheat landraces A5 and A7 exhibited relatively high chlorophyll content, with the control plants (grey bars) generally showing higher values than the inoculated plants (black bars). Landrace B5 showed the most significant difference, where the control plants had an evidently higher chlorophyll content than their inoculated counterparts. Interestingly, landrace C3 showed the opposite trend; AMF-inoculated plants had higher chlorophyll content than the control plants.

2.4.2. Morphological method to quantify AMF colonisation

The quantification of fungal structures colonising the roots provided a clear indication that the AMF inoculation was effective across all wheat landraces. However, drought stress significantly reduced the percentage of AMF colonisation compared to well-watered conditions (p <0.001) (Figure 2.3). Wheat landrace A5 under well-watered conditions, showed the highest root colonisation more than 25%, while drought stress reduced its colonisation to approximately 3%. Landraces B5 and C3 followed a similar trend, with well-watered plants exhibiting higher colonisation rates (about 20%), compared to those under drought stress (less than 5%). Remarkably, A7 showed the lowest root colonisation among the inoculated plants, in both well-watered and drought stress treatments.

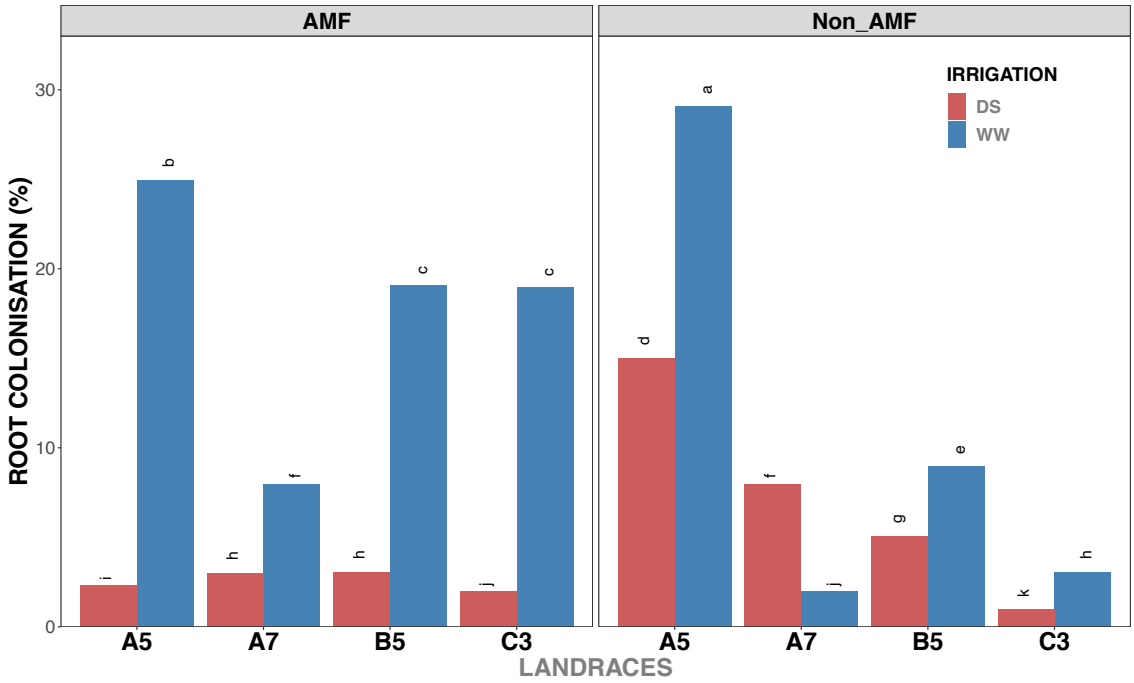


Figure 2.3 | Percentage of root colonisation by AMF (left panel) and other fungi (Non_AMF, right panel) in four wheat landraces (A5, A7, B5, and C3). Fungal colonisation was determined under two irrigation regimes: drought stress (red bars) and well-watered (blue bars) conditions. Different letters show significant differences (p <0.05).

As expected, most of the non-inoculated plants did not show signs of AMF colonisation or were negligible in a few samples (less than 3%) under both irrigation treatments. Moreover, the presence of other fungi rather than AMF was acknowledged and quantified in all four treatments.

In a general context, root colonisation of fungi other than AMF (non-AMF) was substantially lower under well-watered conditions across all landraces, except in wheat landrace A5 that markedly showed higher percentage than those in the AMF-inoculated (A5). Root colonisation in landraces A7, B5, and C3 remained generally below 10%, with only slight differences between drought stress and well-watered conditions.

2.4.3. Molecular method to determine the presence of AMF colonisation

While microscopy provides valuable insights into the physical presence and colonisation patterns of AMF structures within plant roots, molecular methods, such as PCR, can be used to confirm or support their presence.

The initial PCR using the universal ITS1 and ITS4 primers, which target a broad range of fungal species, showed positive results across all samples. This outcome indicated the presence of fungal DNA in the roots, but it is not definitive for AMF alone, as these primers are not exclusive to AMF. It is highly likely that amplification included a mix of AMF and other non-AMF fungi.

To specifically identify the presence of AMF inside wheat roots, a second PCR using the AML1 and AML2 primers, which are more selective for the SSU rRNA gene of AMF, provided more evidence of their presence. The successful amplification and detection of expected band sizes (Table 2.2) in agarose gel electrophoresis suggested that AMF were present in the wheat root samples.

Further validation was completed through the semi-nested PCR with WANDA and AML2 primers, which provided additional confirmation of AMF presence by amplifying a specific region within the SSU rRNA gene. The positive results observed with this technique, confirmed by the presence of expected bands on the agarose gel, reinforced the conclusion that AMF were present in the wheat roots.

2.4.4. Drought stress and aerial dry biomass

The aerial dry biomass (shoots, leaves, and inflorescences without grains) was significantly reduced under drought stress (p <0.001) across all wheat landraces compared to the well-watered treatment, which was expected due to the reduced availability of water.

Drought stress evidently reduced the aerial biomass in all landraces independently of the inoculation treatment (Figure 2.4). However, the difference between inoculation treatments under drought stress was minimal. Notably, landrace A5 showed the highest aerial biomass under drought stress, although AMF inoculation did not significantly enhance biomass under these conditions.

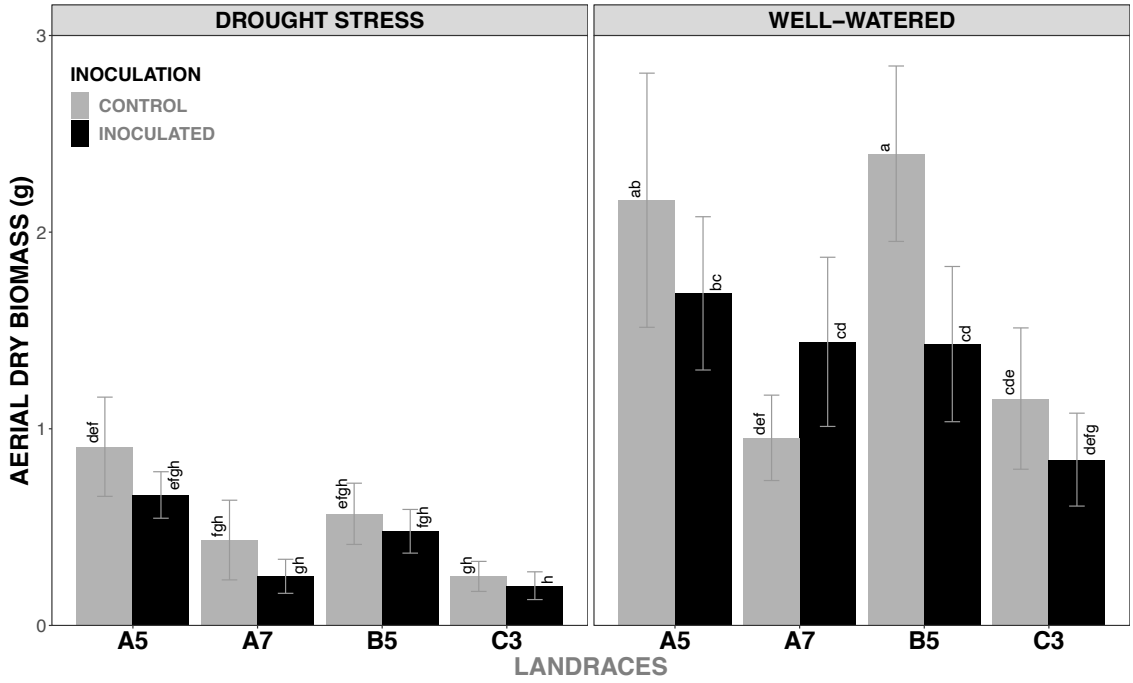


Figure 2.4 | Aerial dry biomass (in grams) of four wheat landraces (A5, A7, B5, and C3) inoculated with AMF or non-inoculated (control) under two irrigation conditions: drought stress and well-watered. Different letters show significant differences (p <0.05).

2.4.5. Drought stress and root dry biomass

Under drought stress, all four wheat landraces showed a general reduction in root dry biomass (p <0.001) compared to their well-watered counterparts. Nevertheless, there was no significant difference between inoculation treatments, except in A7, in which non-inoculated plants exhibited a slight increase in root biomass (Figure 2.5).

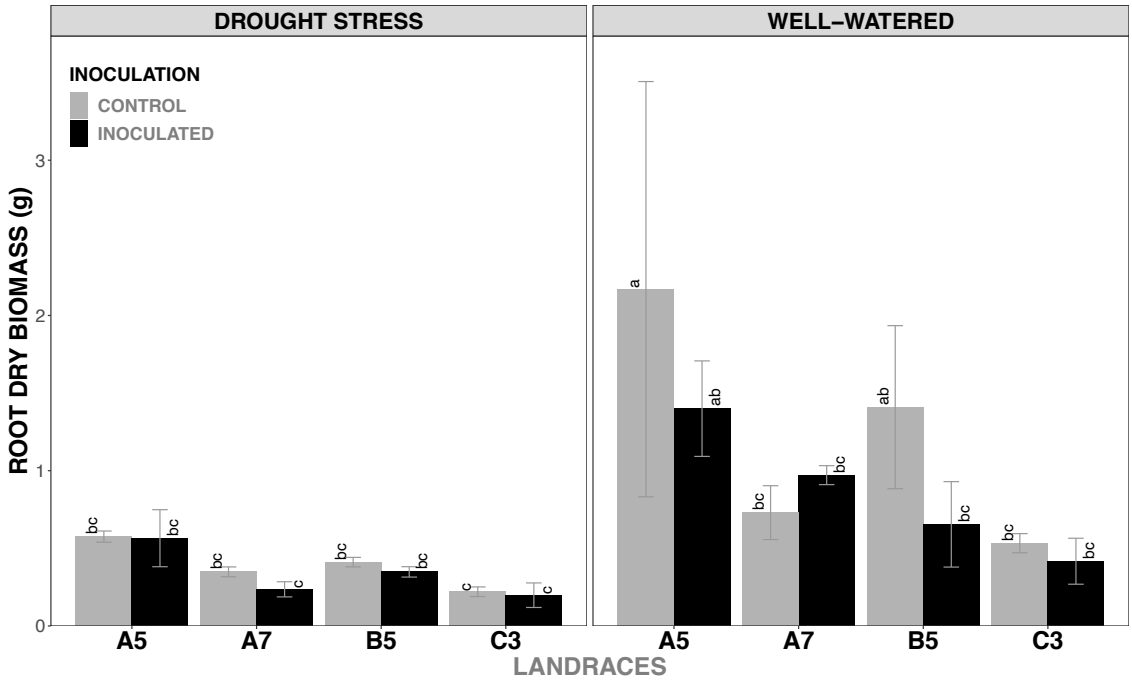


Figure 2.5 | Root dry biomass of four wheat landraces inoculated with AMF or non-inoculated (control) under two irrigation levels: drought stress and well-watered plants. Different letters show significant differences (p <0.05).

2.4.6. Drought stress and plant height

The findings revealed significant differences in plant height among the four wheat landraces under both irrigation conditions, with a significant impact of drought stress (p <0.001). The results further indicated that AMF inoculation under drought did not uniformly influence plant height across all landraces (Figure 2.6).

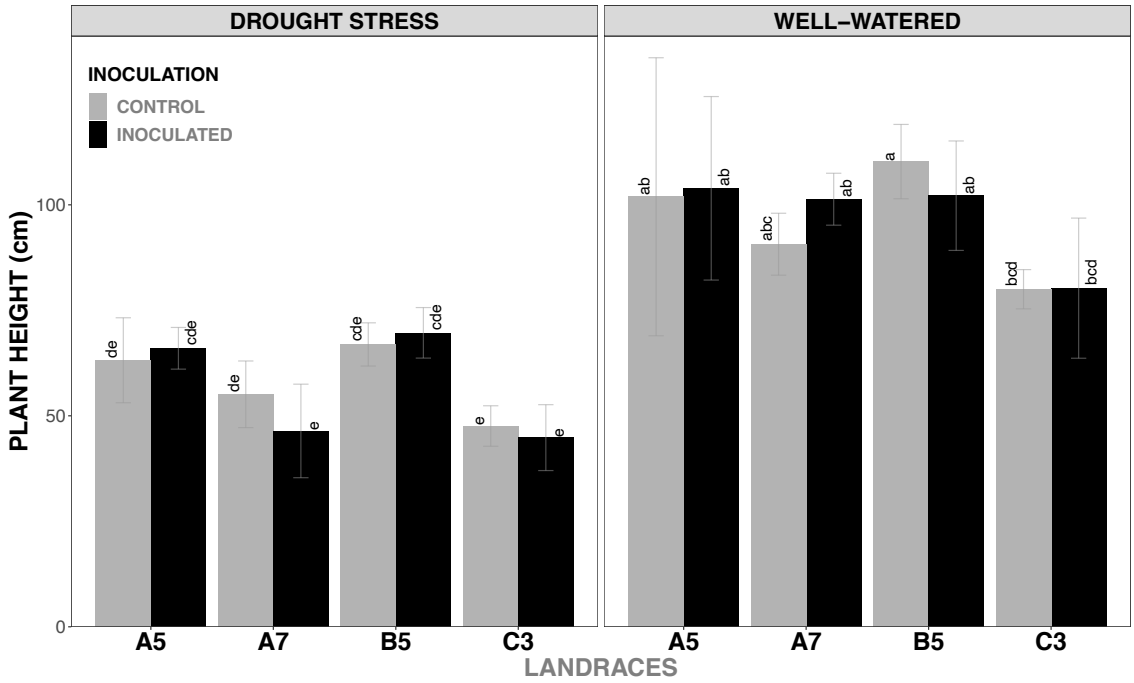


Figure 2.6 | Plant height of four wheat landraces inoculated with AMF (inoculated) or non-inoculated (control) under two irrigation levels: drought stress and well-watered plants. Different letters show significant differences (p <0.05).

Particularly, landrace A5 exhibited a significantly greater height in AMF-inoculated plants under drought, compared to non-inoculated controls, suggesting a potential positive interaction with AMF. Conversely, landrace A7 showed a significant reduction with AMF inoculation under the same condition, which might indicate a genotype-specific response (Figure S2.2).

In the context of analysing the relationship between plant height and dry biomass under drought stress conditions, correlation coefficient of Pearson was used to assess the strength and direction of the linear relationship between these two variables. Interestingly, a positive correlation between plant height and dry biomass (both aerial and root) under drought stress was observed, with a Pearson correlation coefficient of R =0.72 (p <0.001). Nonetheless, under well-watered conditions, this correlation was not strong, implying that factors other than dry biomass accumulation may play a more significant role in plant height when water was not a limiting factor.

2.4.7. Grain yield and yield components

Under drought stress the wheat grain yield was in general lower for all landraces compared to the well-watered treatment (p <0.001), highlighting the negative impact of drought on wheat productivity. Remarkably, wheat landrace A5 showed the lowest grain production per plant with both inoculation treatments exhibiting very low yields, compared with the rest of the landraces (Figure 2.7).

Notably, landrace C3 showed the highest grain yield per plant under drought, nevertheless, a significant reduction in grain yield was observed in AMF inoculated plants, compared to the control.

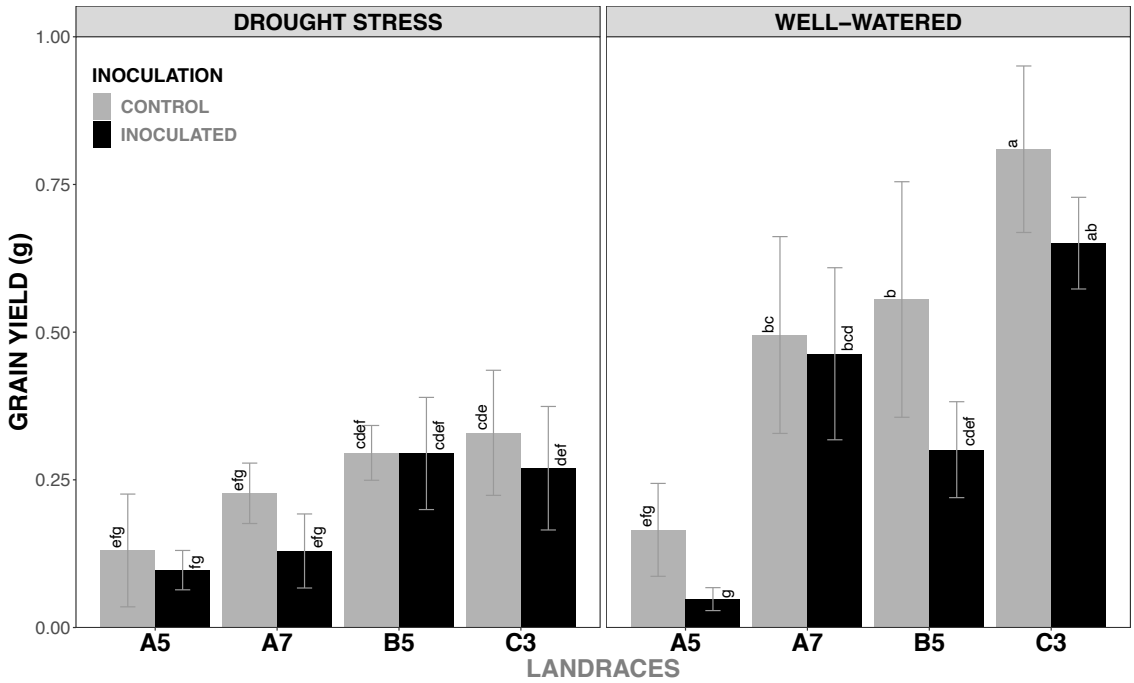


Figure 2.7 | Grain yield of four wheat landraces inoculated with arbuscular mycorrhizal fungi (inoculated) or non-inoculated (control) under two irrigation levels: drought stress and well-watered plants. Different letters show significant differences (p <0.05).

In general, grain yield (per plant) in all wheat landraces under well-watered conditions was significantly higher compared to the drought stress condition, except in A5 which did not show a significant difference with plants under drought stress. Moreover, non-inoculated plants generally produced higher yields per plant

compared to AMF-inoculated ones, with landraces A7, B5, and C3 showing significant differences.

The grain yield per plant in all wheat landraces was found to be directly correlated with the number of grains produced per plant. This relationship remained consistent across both irrigation regimes (Figure 2.8). In this context, the reduction in grain number due to drought conditions, directly contributed to the observed decrease in overall grain yield per plant.

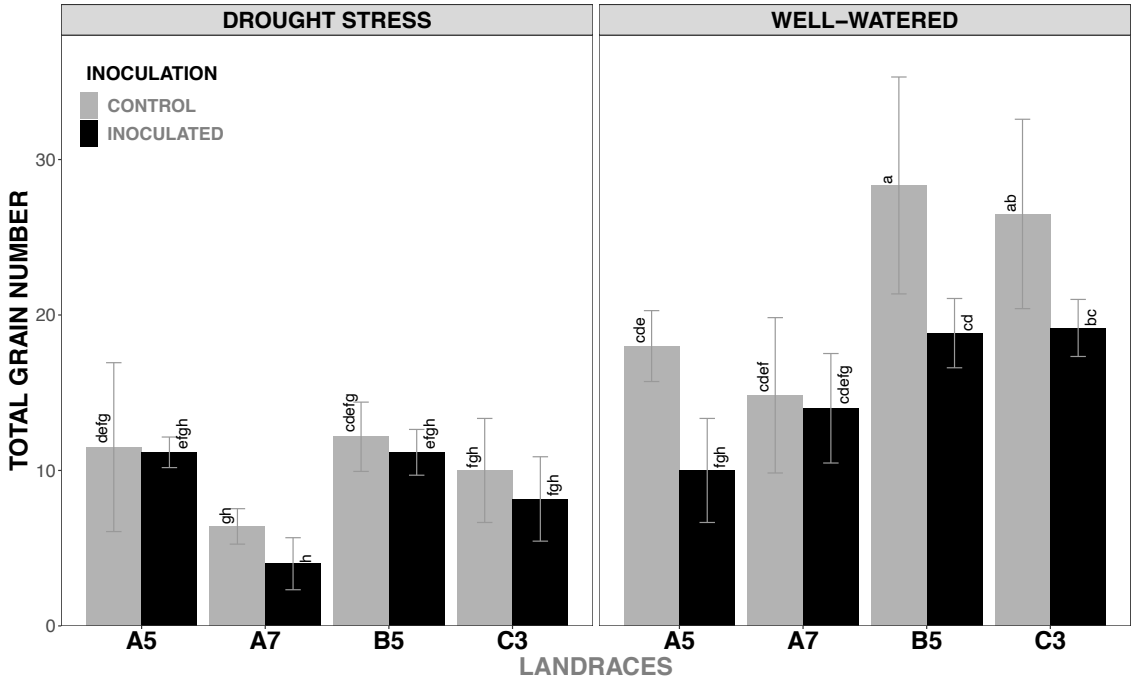


Figure 2.8 | Total grain number per plant of four wheat landraces inoculated with arbuscular mycorrhizal fungi (inoculated) or non-inoculated (control) under two irrigation levels: drought stress and well-watered plants. Different letters show significant differences (p <0.05).

Furthermore, the role of AMF inoculation in influencing grain number appeared to be minimal. In fact, inoculated wheat plants showed statistically lower grain numbers across nearly all landraces.

2.5 Discussion

2.5.1. Leaf chlorophyll increased during drought stress

Wheat plants, whether in natural ecosystems or agricultural conditions, are subject to a range of biotic and abiotic stressors, with drought stress emerging as one of the most critical and prevalent challenges. Drought stress is particularly detrimental to wheat, significantly impairing both developmental processes and grain yield, which are vital to crop productivity and food security (Buchanan et al., 2015; Salehi-Lisar & Bakhshayeshan-Agdam, 2016).

The impacts of drought are profound, as water shortage affects morphological, physiological, and nutritional traits of plants especially water content, photosynthetic pigment, stomatal conductance, and phosphorus and nitrogen absorption, leading to restricted growth and reduced crop production. This makes drought the predominant abiotic stress affecting wheat (Augé et al., 2014; Salehi-Lisar & Bakhshayeshan-Agdam, 2016; Symanczik et al., 2018).

Due to the increasing incidence and severity of drought events under the influence of climate change, understanding the mechanisms by which drought stress affects wheat landraces growth and yield, is essential. Furthermore, developing effective strategies to enhance drought tolerance through the use of wheat landraces, which are more diverse genetically, is crucial for ensuring global food security.

It is well-documented that chlorophyll content serves as a reliable indicator of plant health and photosynthetic activity under drought stress conditions (Barnabás et al., 2008; Anli et al., 2020; Monteoliva et al., 2021; Yang et al., 2023; Ishfaq et al., 2024). In the present experimental project, chlorophyll levels were measured at both the vegetative and flowering stages of wheat development. Notably, during the vegetative stage, no significant differences in chlorophyll content were observed between treatments, even under drought stress. This suggests that, at this early stage, wheat landrace plants maintained their photosynthetic capacity despite water stress (Zhao et al., 2020; Maazouz, et al., 2023). However, a marked contrast

was observed during the flowering stage, at this critical developmental phase, chlorophyll levels varied significantly across treatments. The significant increase in chlorophyll levels under drought stress could be interpreted as an adaptive response by the wheat plants.

Under drought stress, leaves typically reduce their chlorophyll concentration, a process often driven by the production of singlet oxygen, which can result in the destruction of chlorophyll (Ommen et al., 1999; Barnabás et al., 2008; Jaleel et al., 2009; Rolando et al., 2015; Hasanuzzaman et al., 2019). The results in this experimental project agree with a similar increase in leaf chlorophyll reported in different studies involving various plants such as wheat (Hamada, 1996), *Solanum tuberosum* L. (Yactayo et al., 2013; Ramírez et al., 2014; Rolando et al., 2015), and the grass *Bouteloua gracilis* (García-Valenzuela et al., 2005) under drought and osmotic stress, respectively. In these studies, an increase in leaf greenness was observed (one week) after the initiation of water restriction, persisting for several weeks. Additionally, Mathur et al. (2019) reported that wheat plants inoculated with AMF also displayed higher chlorophyll content when subjected to drought stress, compared with non-inoculated treatments, suggesting an AMF mechanism for enhancing the ability of the plant to maintain or increase chlorophyll levels during water deficit.

Diverse authors (Bojović & Marković, 2009; Parry et al., 2014; Rolando et al., 2015; Bansal & Srivastava, 2020), suggest that the increase in chlorophyll concentration is a consequence of reduced leaf growth under drought conditions, as the ones observed in this experimental project (supplementary Figure S2.2), which concentrates chlorophyll within a smaller leaf area. This response may serve as a strategy (under drought), to maintain chlorophyll levels, and potentially sustain photosynthetic activity despite limited water availability.

2.5.2. AMF colonisation

The reduction in root colonisation in four all wheat landraces under drought stress might be attributed to the negative impact of water deficit. Diverse studies have

investigated the effects of drought on wheat (Li et al., 2005; Liu et al., 2016; Abid et al., 2018; Baslam et al., 2018); it was found that drought stress significantly reduced AMF colonisation by nearly 50% compared to well-watered control plants. This was in accordance with the results found in this experimental project, where the percentage of root colonisation dropped significantly, more than 50% in all wheat landraces, compared with their well-watered counterparts.

Diverse studies have attributed this reduction in the percentage of root colonisation to factors such as, decreased root growth, altered root exudate composition (which delayed or suppressed AMF establishment), impaired metabolism of AMF (affecting their ability to form mycelial networks), lower soil moisture, and increased competition for resources (Bowles et al., 2016; Li et al., 2017; Wu et al., 2019; Pellegrino et al., 2020; Zhou et al., 2020).

As previously stated, inoculated wheat plants received soil containing AMF propagules as live inoculum. In addition to AMF, other fungi (Figure 2.3, non-AMF) colonised the root systems of all four wheat landraces. Conversely, control plants (non-inoculated) exhibited either no fungal colonisation or very low levels, ranging from approximately 1 to 3% (for both AMF and non-AMF). Given that control plants were sown in a substrate containing sterilised soil supplemented with a filtered soil solution, it can be hypothesised that this resulted in a more favourable wheat (soil and root) microbiome. This environment potentially harboured a greater proportion of beneficial rhizobacteria and/or a negligible number of fungi. Consequently, this altered microbial composition might have influenced the growth and development of the control wheat plants compared to the AMF-inoculated treatments, irrespective of the irrigation regime.

On the other hand, AMF inoculated wheat plants under well-watered conditions, showed a relatively low root colonisation (<30 %), likely due to a reduced need for the symbiotic benefits provided by AMF. In well-watered environments, plants can normally access water and nutrients from the soil without relying on AMF, consequently, the plant may allocate fewer resources (carbon) to support AMF colonisation. This reduced dependency results in lower AMF colonisation compared

to drought conditions, where the symbiotic relationship becomes more crucial for plant survival and growth (Subramanian et al., 2006; Pons and & Müller, 2022).

However, the timing of calculating the percentage of AMF colonisation in plants, particularly in wheat, is important for accurately assessing the symbiotic relationship and its impact on plants. In this experimental project, the percentage of root colonisation was carried on at the end of the plant life cycle (Z99), which could give a narrow perception of the dynamics and benefits in the symbiotic relationship. For instance, in a study by Al-Karaki & Al-Raddad (1997), AMF colonisation was measured at different stages to understand its effect on wheat growth under drought stress. Similarly, another study by Al-Karaki (2006) suggests that measuring colonisation at key developmental stages, such as the grain filling stage, can provide a perception of how AMF influences nutrient uptake and stress tolerance.

2.5.3. Height and dry biomass among wheat landraces

Drought stress significantly reduced the height of all four wheat landraces, irrespective of AMF inoculation (Figure S2.2). This finding aligns with the results reported by Zhao et al. (2020) and Fiorilli et al. (2022). Moreover, the positive correlation observed between wheat height and irrigation treatments was attributed to the decrease in total dry biomass, encompassing leaves, shoots, and roots. The reduction in aerial and root (total) dry biomass affected the growth and development of all wheat landraces, regardless of the inoculation treatment. In comparison to control treatments, all well-watered wheat landraces exhibited higher total dry biomass, resulting in greater stature.

2.5.4. Grain yield and yield components under drought stress

Numerous studies have investigated the effects of AMF colonisation on wheat grain yield and number of grains under drought stress conditions. These studies have demonstrated that, under drought conditions, inoculation with AMF can significantly improve grain yield compared with non-inoculated control plants.

However, in this experimental project, grain yield per plant and the number of grains per plant decreased significantly as a consequence of drought stress. These findings align with those reported by Sendek et al. (2019), who observed that AMF colonisation did not consistently mitigate the detrimental effects of drought in barley, including its impact on grain yield.

2.6 Appendix S2

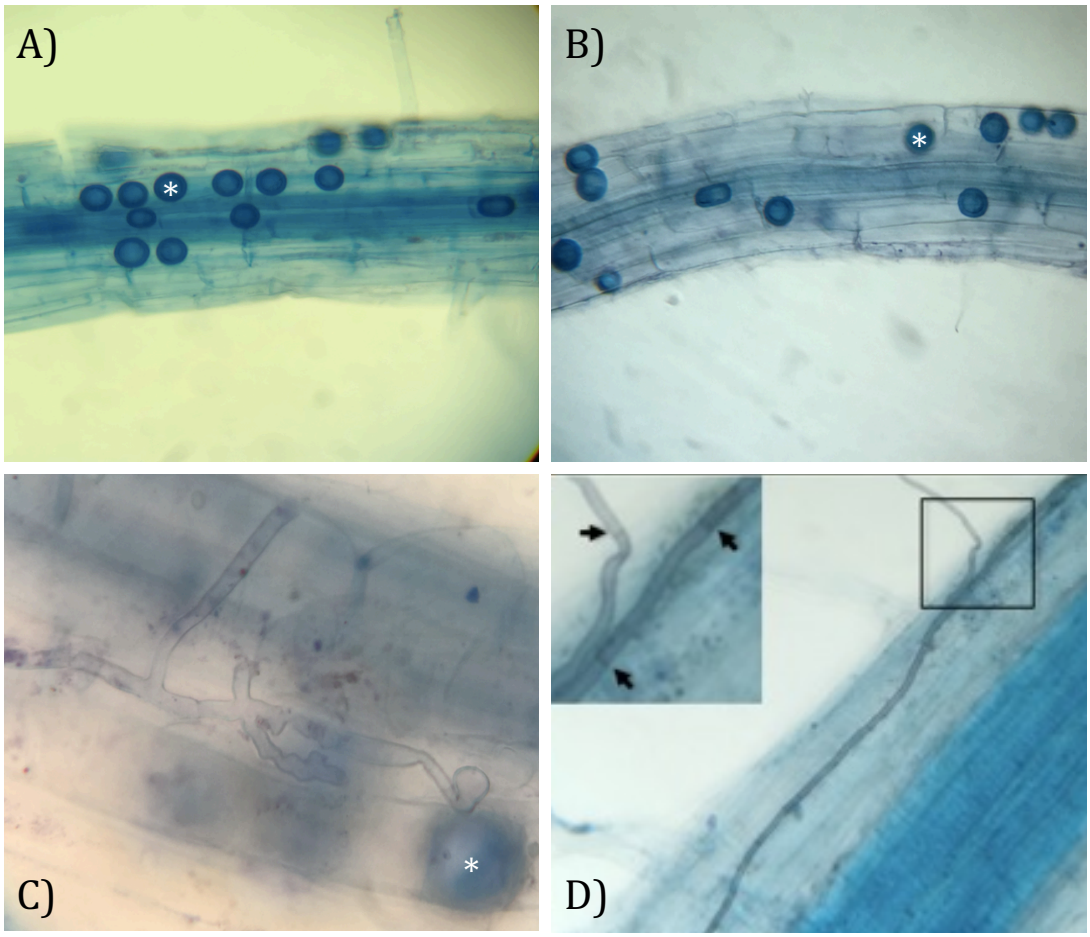


Figure S2.1 | Fungal structures observed in stained wheat roots. The pictures show representative fungal (AMF and non-AMF) structures found in the different wheat landraces. A) and B) intraradical spores, C) intraradical hyphae, and D) hyphae of non-AMF, they are differentiated by the presence of septa (cell divisions), which are indicated with arrows. Asterisks denote spores.

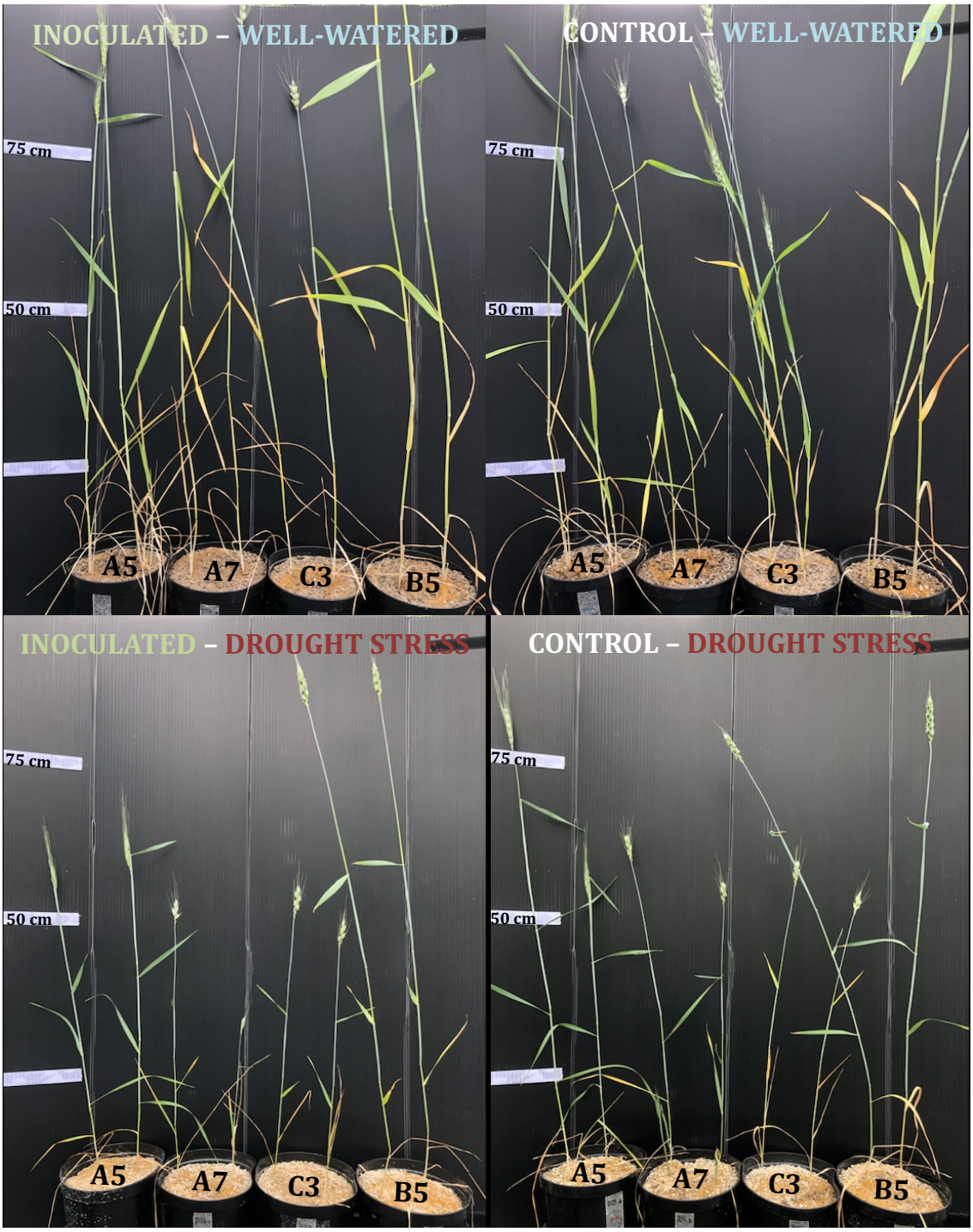


Figure S2.2 | Representative photograph of the four wheat landraces (A5, A7, C3, and B5), subjected to two inoculation treatments: inoculated with AMF (inoculated) or non-inoculated (control), under two irrigation levels: drought stress and well-watered plants. Height intervals (in cm) are shown on the left side of each treatment.

Chapter 3. Identification of fungal communities in four wheat landraces employing Illumina MiSeq sequencing platform

3.1 Introduction

In natural and agricultural environments, rhizospheric microorganisms establish diverse relationships with plants, generally classified as mutualistic or parasitic, both of which are critical for maintaining homeostasis in the soil ecosystem (Gqozo et al., 2020). Most plants recruit beneficial (mutualistic) rhizospheric microorganisms, such as AMF, endophytic fungi, nitrogen-fixing bacteria, PGPR, and protozoa, which play a fundamental role in plant health and nutrition. Conversely, parasitic or pathogenic microorganisms can cause plant diseases (Smith & Read, 2008; Gqozo et al., 2020).

Besides AMF, endophytic fungi can reside partly or entirely within healthy plant tissues throughout their life cycle without causing any harm. Endophytic fungi belong to diverse ecological groups, broadly classified as clavicipitaceous fungi (associated with grasses) and non-clavicipitaceous fungi (found in plants other than grasses) (Natsiopoulos et al., 2024). Although they do not form mycelia, endophytic fungi play an important role in promoting plant health and growth. Their benefits include enhanced nutrient uptake, root system expansion, plant elongation, among others (Liu et al., 2022).

For instance, certain endophytic species (*Trichoderma*) have been shown to produce antimicrobial compounds that directly inhibit the growth of soil-borne pathogens that compete for space within the plant tissues and nutrients, whilst others stimulate the production of defence-related enzymes and metabolites in the wheat plant. It has also been documented that inoculation of AMF and endophytic fungi could significantly alleviate the adverse effects of abiotic stresses such as drought and salinity (Pozo & Azcón-Aguilar, 2007; Ripa et al., 2019; Akram et al., 2023; Natsiopoulos et al., 2024; Zhang et al., 2024).

Fungal diversity can influence the microbial community structure in the rhizosphere, which is vital for plant interactions that promote plant health and growth. Therefore, the study of the fungal population of the wheat microbiome is crucial to understand the interactions between AMF, endophytic, and pathogenic fungi, and how different environmental conditions may influence or regulate plant performance and (AMF) fungal colonisation (Bever et al., 2012).

For studying fungal communities in wheat, the MiSeq platform offers several advantages; its high-resolution sequencing allows for the identification of a wide range of fungal taxa, including species that are less abundant and might be unnoticed by traditional methods. This is particularly valuable for studying AMF, which are often difficult to identify and quantify using conventional morphological and biochemical techniques (www.illumina.com; Piro, 2020; Victorino et al., 2020).

The capacity of the MiSeq platform to analyse multiple samples simultaneously facilitates large-scale studies of fungal communities across different wheat varieties or landraces, growth stages, or even environmental conditions. Diverse studies have demonstrated the advantages of MiSeq sequencing in elucidating the fungal communities associated with wheat. For instance, in a study by Schlatter et al. (2017), the Illumina MiSeq platform was used to sequence the 18S rRNA gene of AMF in wheat roots. The sequencing data provided insights into the diversity of AMF associated with wheat and how these communities might influence plant health by suppressing diseases. The use of MiSeq allowed the detection of a broad range of AMF species, highlighting its utility in exploring the complex interactions within the rhizosphere.

Comparably, the study by Stefani et al. (2020) investigated whether different durum wheat cultivars recruited specific AMF communities from indigenous soil populations under field conditions. Sequencing of the 18S rRNA gene using the MiSeq platform, the study identified amplicon sequence variants belonging to the Glomeromycotina subphylum across the different samples. This research emphasised the efficacy of MiSeq sequencing in providing detailed insights into AMF communities in agricultural systems.

Furthermore, a study by Marrassini et al. (2024) investigated the influence of wheat genotypes and AMF inoculation on wheat productivity and quality. Using MiSeq sequencing, the researchers found that both wheat genotype and AMF inoculation significantly affected the composition of root-associated AMF communities. These changes, in turn, impacted wheat yield and the quality of processed wheat products.

Therefore, understanding the fungal communities in wheat landraces is particularly significant because landraces often possess unique genetic traits that can influence their interactions with fungal communities. Wheat landraces are genetically diverse, and they may harbour both beneficial and pathogenic fungal interactions differently than modern (high yielding) wheat varieties (Zhu et al., 2000; Gianinazzi et al., 2010). Hence, understanding these interactions can provide insights into how to manage fungal communities, particularly AMF to enhance wheat productivity, especially in the face of environmental stresses such as drought.

3.1.1. Aims and objectives

The aim of this research project was to identify and elucidate the interactions among fungal communities, including AMF and general fungi (both mutualistic and parasitic), in four spring wheat landraces under two distinct irrigation regimes. This was accomplished through next-generation sequencing analysis. Furthermore, the study aimed to investigate the potential influence of these fungal communities on plant health and productivity by examining the interactions and changes of diverse fungal taxa.

The objectives of this study were as follows: (a) to systematically characterise the fungal community composition within four spring wheat landraces (A5, A7, B5, and C3) under two distinct irrigation regimes, utilising two sets of primers: ITS for the identification of general fungal taxa, and WANDA-AML2 specifically targeting AMF, via next-generation sequencing on the Illumina MiSeq platform. For this objective it was hypothesised that the diversity and composition of both general fungal and AMF communities in wheat roots will differ significantly between specific wheat landraces, and distinct fungal associations in response to drought stress.

(b) To conduct a comparative analysis of fungal community structures across four spring wheat landraces, focusing on the changes revealed by sequencing with ITS and WANDA-AML2 primers, and (c) to investigate whether associations between fungal community composition and abundance are correlated with plant morphological and physiological parameters in spring wheat landraces.

The hypotheses for these objectives were that (i) the fungal community composition and abundance, as revealed by ITS and WANDA-AML2 primers, will exhibit both shared and unique taxa across wheat landraces, and (ii) differences based on water regimes, potentially will be correlated with plant growth and stress responses.

3.2 Materials and methods

3.2.1. Illumina MiSeq sequencing

DNA samples extracted from the roots of four wheat landraces (A5, A7, B5, and C3) from the experiment in Chapter 2 were used for sequencing using the Illumina MiSeq platform, samples for AMF and ITS were assigned specific names for all four treatments (Table 3.1).

Table 3.1 Assigned sample names for the root DNA from four wheat landraces, under two inoculation levels and two irrigation regimes (WW, well-watered, DS, drought stress).

SAMPLE ITS	SAMPLE AMF	INOCULATION	IRRIGATION	LANDRACE
gA1	**wA1**	Inoculated	WW	A5
gA2	**wA2**	Inoculated	WW	A5
gA3	**wA3**	Inoculated	WW	A7
gA4	**wA4**	Inoculated	WW	C3
gA5	**wA5**	Inoculated	WW	B5
gA6	**wA6**	Inoculated	WW	B5
gN1	**wN1**	Control	DS	A5
gN2	**wN2**	Control	DS	A5
gN3	**wN3**	Control	DS	A7
gN4	**wN4**	Control	DS	C3
gN5	**wN5**	Control	DS	C3
gN6	**wN6**	Control	DS	B5
gR1	**wR1**	Inoculated	DS	A5
gR2	**wR2**	Inoculated	DS	A5
gR3	**wR3**	Inoculated	DS	A7
gR4	**wR4**	Inoculated	DS	C3
gR5	**wR5**	Inoculated	DS	C3
gR6	**wR6**	Inoculated	DS	B5
gV1	**wV1**	Control	WW	A5
gV2	**wV2**	Control	WW	A5
gV3	**wV3**	Control	WW	A7
gV4	**wV4**	Control	WW	C3
gV5	**wV5**	Control	WW	C3
gV6	**wV6**	Control	WW	B5

Before sequencing, SSU fragments (AMF samples) were amplified with two pairs of primers (Table 3.2). In the first step, samples were amplified using the forward primer AML1 and the reverse primer AML2 primer, while a semi-nested PCR was performed using the forward primer WANDA-ill and the reverse primer AML2-ill were used in the second step, PCRs were amplified as described in Chapter 2.

Table 3.2 Primer pairs used in this study. (F), Forward primer; (R), Reverse primer. SSU, small subunit rRNA gene; ITS, internal transcribed spacer. The Illumina primer design was as follows: 5' (F or R adaptor-F or R primer) 3'.

PRIMER PAIR	SEQUENCE (5' → 3')	SIZE (BP)	TARGET REGION	REFERENCE
AML1 (F)	ATC AAC TTT CGA TGG TAG GAT AGA	800	SSU	Lee et al., 2008
WANDA (F)	CA GCC GCG GTA ATT CCA GCT	550-600		Dumbrell et al., 2011
AML2 (R)	GA ACC CAA ACA CTT TGG TTT CC			Lee et al., 2008
Illumina adapter (F)	TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG ANN NHN NNW NNN H	-	SSU	Dumbrell et al., 2011
Illumina adapter (R)	GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G	-		Lee et al., 2008
ITS1 (F)	TCC GTA GGT GAA CCT GCG G	580-750	ITS	White et al., 1990
gITS7 (F)	GT GAR TCA TCG ART CTT TG	350-400		Ihrmark et al., 2012
ITS4 (R)	TC CTC CGC TTA TTG ATA TGC			White et al., 1990

The ITS samples followed the same protocol for sequencing; in a first step, samples were amplified using the forward primer ITS1 and the reverse primer ITS4 primer, while in the second step, a semi-nested PCR was performed using the forward primer gITS7 and the reverse primer ITS4-ill.

The quality of all PCR products was examined by gel electrophoresis through a 1.5% agarose gel in 0.5 × TBE buffer, then purified with magnetic beads (Agencourt AMPure® XP, Beckman Coulter, USA) following the protocol of the manufacturer. Then, DNA concentration was quantified by fluorescence with the Quant-iTTM dsDNA High-Sensitivity Assay Kit in a Qubit® 2.0 Fluorometer (Invitrogen by Thermo Fisher Scientific, Italy), following the protocol of the manufacturer.

The cleaned and quantified amplicons of each library were adjusted in an equimolar ratio (10 ng μL^{-1}) for the addition of dual-index barcodes using the Nextera® XT

DNA library preparation kit (Illumina Inc., CA, United States), and the generated metabarcoding libraries were sequenced on an Illumina MiSeq sequencer (2 × 300 bp paired-end reads) by the Bioscience Technology Facility at the University of York (UK).

3.2.2. Pipelines for analysing the Illumina MiSeq reads

For the ITS region, the demultiplexed Illumina sequencing data (FASTQ files, i.e., FASTA files with integrated quality information) were processed using the QIIME2 (Quantitative Insights Into Microbial Ecology, v. 2018.11; http://qiime2.org/) pipeline and its associated plugins (the script is listed in Supplemental Table S3.1) (Bolyen et al., 2019).

The UNITE database (https://unite.ut.ee; Abarenkov et al., 2010; Nilsson et al. 2018) was used to train the classifier. Subsequently, primers were trimmed from sequences using cutadapt (Martin, 2011). The sequences were then denoised using DADA2 (Divisive Amplicon Denoising Algorithm, v. 1.24.0; Callahan et al., 2016) and taxonomically classified employing the latest release of the trained classifier (UNITE QIIME release v. 9.0; 2022-10-16) as reference sequences for the identification of the target ITS region (Abarenkov et al., 2022).

For AMF identification using the 18S rRNA gene, sequences were processed using the DADA2 package in RStudio (the script is listed in Supplemental Table S3.2). The pipeline began with filtering the demultiplexed fastq files, followed by dereplication and denoising. Afterward, chimera or bimera sequences (two-parent chimeras) were identified and removed from the denoised output. Then, forward and reverse paired reads were merged (if they overlapped exactly); alternatively, the forward reads could be used alone in the pipeline. For the taxonomic classification and subsequent identification of the AMF, it has been established that different databases can be used. In this study, the obtained sequences were classified using the SILVA database (https://www.arb-silva.de; Quast et al., 2013). Representative sequence reads were then BLASTed against the specialised MaarjAM database for

AMF (https://maarjam.ut.ee; Öpik et al. 2010) to corroborate that they matched (at least 97%) with the AMF classification.

3.2.3. Fungal taxa based on their trophic modes: FUNGuild

Additionally, the taxonomic file was subjected to an analysis to assign functional trophic guilds using the open-source software FUNGuild (v. 1.0; Nguyen et al., 2016). This tool classifies the OTUs/ASVs into three categories: saprotrophs, symbiotrophs, and pathotrophs, according to the online FUNGuild database containing taxonomic and functional metadata.

These trophic modes are broadly defined as they reflect the primary feeding habits of fungi: (1) Saprotrophs receive nutrients by breaking down dead host cells. (2) Symbiotrophs receive nutrients by exchanging resources with host cells. (3) Pathotrophs (including phagotrophs) receive nutrients by harming host cells.

The analysis assigns functions based on genus and species levels, with confidence levels described as highly probable, probable, and possible. A limitation of the FUNGuild assignment is that some fungi can be categorised in more than one guild. To address this, a confidence category is provided which, when combined with ecological information, allows for the assignment of a proper guild (Nguyen et al., 2016).

3.2.4. Statistical analyses

The alpha diversity richness was assessed using observed richness, the Shannon index (Shannon, 1948) and Simpson index (Simpson, 1949) for the amplicon sequence variants (ASVs). Beta diversity was measured using the Bray-Curtis distance matrix (Roger Bray & Curtis, 1957). Differences in alpha diversity were analysed using ANOVA, whilst beta diversity was analysed using the Bray-Curtis dissimilarity and permutational multivariate analysis of variance (PERMANOVA)

with 999 permutations. All statistical analyses were carried out using RStudio software (v. 4.2.2).

3.3 Results

3.3.1. ITS sequencing data analysis

The quality of the ITS sequencing data was thoroughly assessed using bioinformatic tools within the QIIME2 pipeline. This evaluation revealed that the R2 (reverse) reads generally exhibited lower quality scores compared to the R1 (forward) reads. Such a discrepancy in quality between forward and reverse reads is a common occurrence in Illumina sequencing datasets, often attributed to the degradation of reagents and enzymes over the course of the sequencing run.

To prevent the potential impact of these lower-quality reads on the results, a two-step approach was implemented. Firstly, the reads were trimmed based on their average quality scores. This process involves removing the lower-quality portions of the reads, typically found at the 3' end, to retain only the high-quality sequences for further analysis. Secondly, given the particularly low quality of the R2 reads, a decision was made to omit these reads entirely during the denoising step, prior to merging with their R1 counterparts. This strategy, while resulting in a loss of some sequence information, was necessary to prevent the introduction of errors and to enhance the overall accuracy of the analysis (Callahan et al., 2016; Ramakodi, 2021).

3.3.2. Fungal diversity associated with the wheat landraces

The analysis of the sequenced ITS region revealed the presence of nine fungal phyla across the wheat root samples. Relative abundance showed the proportional distribution of different fungal phyla in each sample, among these, Ascomycota, Basidiomycota, and Mortierellomycota appeared as the dominant ones. The remaining six phyla Glomeromycota (subphylum Glomeromycotina), Chytridiomycota, Rozellomycota, Mucoromycota, Monobepharomycota, and Olpidiomycota exhibited less uniform distribution, with notably lower read numbers in some samples (Table 3.1). Additionally, a group of sequences could not be assigned beyond the Kingdom level and were consequently classified as 'Other'.

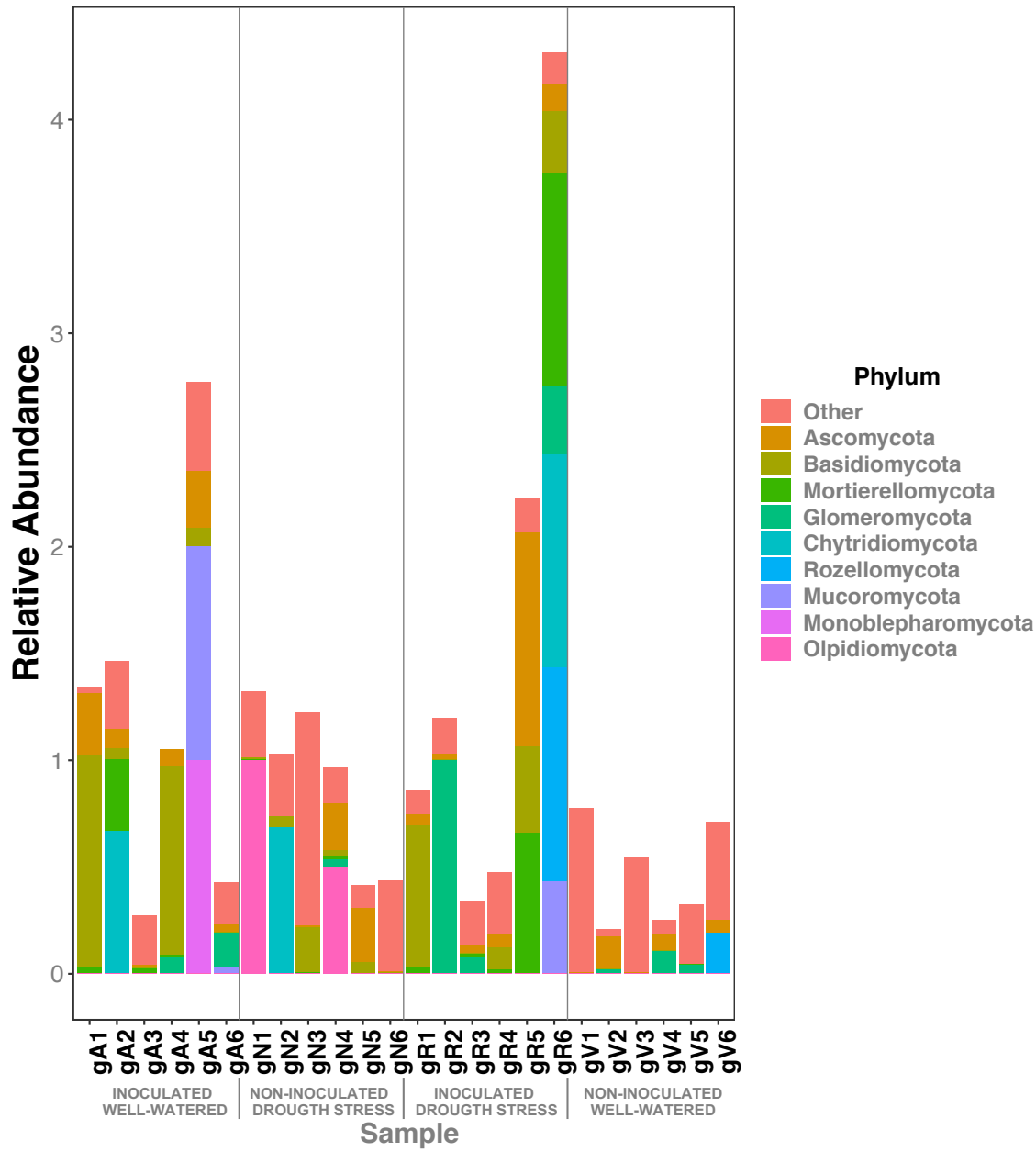


Figure 3.1 | Relative abundances of the fungal phyla identified with ITS in four different wheat root landraces, under four treatments: gA, inoculated, well-watered; gR, inoculated, drought stress; gV, control, well-watered; gN, control, drought stress.

From these nine phyla, a total of 870 ASVs were identified across the wheat root samples. Based on relative abundance, Ascomycota represented the majority, accounting for 50% of the total ASVs and encompassing 60 families. Basidiomycota followed, comprising 13% of ASVs across 44 families. Mortierellomycota, despite its dominance, showed limited diversity with only 1.5% of ASVs belonging to a single family. Glomeromycota, where AMF are found, only represented 1.2% of ASVs across four families. The remaining phyla constituted less than 1% of the total ASVs.

The Glomeromycota phylum showed a lower representation than initially anticipated since colonisation of AMF was observed (and quantified) in the roots. The sequencing analysis identified four families within this phylum, with their distribution varying across treatments and wheat landraces. Genera Glomus, Archaeospora, and Funneliformis were identified under drought stress treatment in the wheat landraces A5, A7, and B5, respectively. Additionally, under well-watered conditions, wheat plants in landrace B5 and C3 harboured genera Funneliformis and Paraglomus, respectively.

3.3.3. Absolute abundance

The absolute abundance analysis quantified the number of sequences attributed to each phylum. It revealed significant differences between treatments. Overall, the inoculated treatments (designated as gA and gR samples) harboured a statistically higher fungal community diversity compared to the non-inoculated control treatments (designated as gN and gV samples) (Figure 3.2).

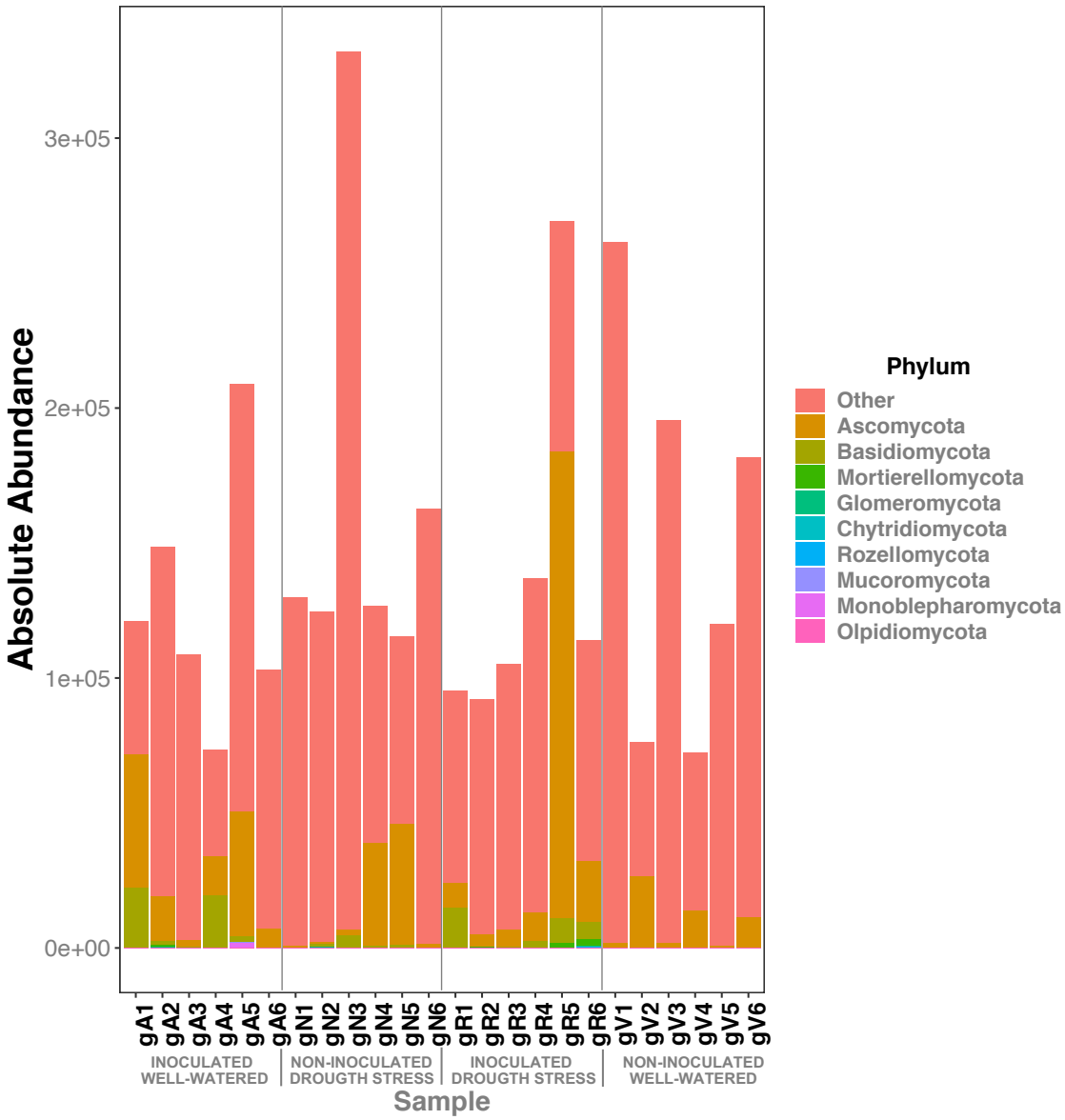


Figure 3.2 | Absolute abundances of the ITS fungal phyla in four different wheat root landraces, under four treatments: gA, inoculated, well-watered; gR, inoculated, drought stress; gV, control, well-watered; gN, control, drought stress.

Drought stress influenced the fungal abundance across the wheat landraces, with the most pronounced effect noted in the wheat landrace C3 when compared to the control conditions. Landrace C3 demonstrated the highest plasticity in its fungal associations under drought stress conditions, particularly among the Ascomycota, Basidiomycota, and Glomeromycota phyla. This was followed by landraces B5, A5, and A7, with a lesser impact.

3.3.4. Ecological functions or guilds

Guilds were successfully assigned to 45% of the total ASVs (Figure S3.1), in which Ascomycota and Basidiomycota were the most dominant phyla within the fungal communities of the studied samples, with a diverse range of trophic modes. Saprotrophy was a predominant mode, especially in Ascomycota and Basidiomycota, reflecting the role of fungi in nutrient cycling and organic matter decomposition.

Furthermore, the Glomeromycota phylum consisted exclusively of symbiotrophs, including the AMF was low. The phylum Mortierellomycota exhibited much lower counts, although it was structured by saprotroph-symbiotrophs. Chytridiomycota, Mucoromycota, and Olpidiomycota had negligible representation, with small counts in only one or two trophic modes.

3.3.5. Alpha and beta diversity

Alpha diversity metrics were employed to compare the fungal diversity of wheat root samples across different treatments. ANOVA revealed a significant difference in alpha diversity metrics between inoculation treatments (p= 0.0252). Inoculated samples exhibited higher fungal diversity compared to the control treatments (Figure 3.3A).

Conversely, drought stress (irrigation treatment) did not influence the alpha diversity metrics. Drought resulted in a negligible increase in species richness but did not strongly affect diversity or evenness in the fungal communities compared to well-watered conditions (Figure 3.3B). Furthermore, there were no significant interactions observed between wheat landraces (landrace variable) and other experimental factors in terms of their effect on fungal diversity. Landrace B5 showed the highest richness and A5 the lowest, the diversity followed a similar trend, and the evenness of the fungal community showed variation across landraces (Figure 3.3C).

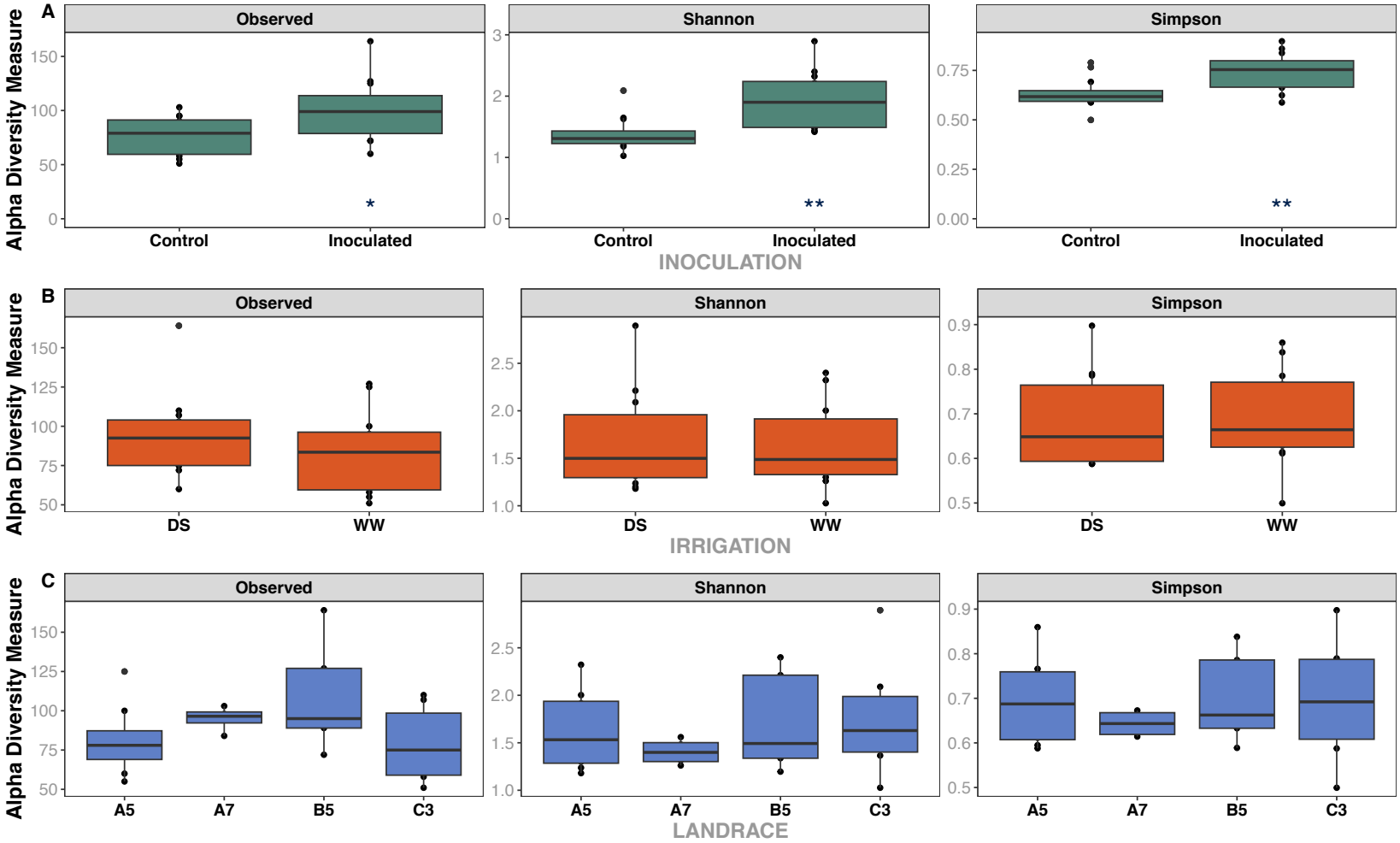


Figure 3.3 | Boxplots of the alpha diversity metrics (Observed ASVs, Shannon index and Simpson index) between treatments: A) Control and Inoculated samples, B) irrigation treatment (DS, drought stress and WW, well-watered) and C) four wheat landraces used in this study. Asterisks indicate statistical significance (*: $p < 0.05$; **: $p < 0.01$). Boxplots represent medians (thick line), interquartile ranges (box), and 95% confidence intervals (error bars).

The beta diversity of the fungal communities associated with wheat roots was assessed using Bray-Curtis dissimilarity analysis. A two-dimensional Principal Coordinate Analysis (PCoA) plot was generated to visualise the fungal community composition among samples.

The analysis revealed a significant effect of wheat landrace on fungal community composition (p= 0.001). Comparably to the alpha diversity metrics, the inoculation treatment exhibited statistically significant effects on the fungal communities (p= 0.05). The PCoA plot showed distinct clustering patterns (Figure 3.4), with samples from the same wheat landrace and inoculation treatment grouping more closely together, correlating with the statistical findings. Notably, drought stress (irrigation treatment) did not exhibit significant interactions with any of the other experimental variables in terms of its effect on fungal community composition.

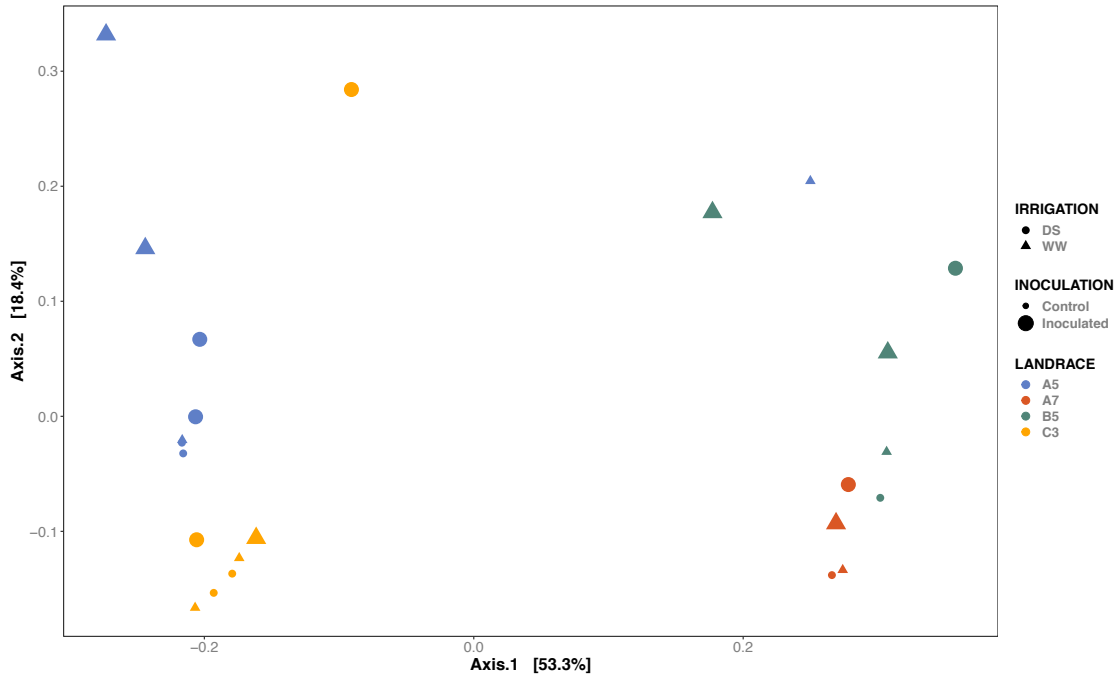


Figure 3.4 | The Principal Coordinate Analysis (PCoA) plot shows around 71% of the total variance between samples, in both first and second axis, samples followed a pattern mainly by landrace (different colours) and to a lesser degree, inoculation treatment (size) that accounted for the differences in the fungal community compositions.

3.3.1. SSU sequencing data analysis

The resulting reads of the 18S rRNA gene were analysed following the DADA2 pipeline and classified using established databases. However, this approach proved unsuccessful in accurately identifying and taxonomically classifying the AMF. The sequences were broadly classified within the Mucoromycota phylum.

Only a small percentage (less than 0.5%) of the reads were positioned within the order Glomerales, family Glomeraceae, and categorised at genus Glomus. This classification also included samples from the non-inoculated treatment (wV and wN). The inconsistency in the sequencing results of the samples suggested potential issues in the pipeline analysis or concerns regarding sequence quality. To address these inconsistencies, various parameters in the bioinformatic analysis were adjusted, including modifications to the trimming length based on read quality and the utilisation of alternative software for sequence analysis. Regarding sequence quality, the reads were subjected to BLAST analysis with the International Wheat Genome Sequencing Consortium (IWGSC). It resulted in a significant high proportion of the reads, approximately 70%, aligned with the IWGSC. Despite efforts, no significant improvements in classification accuracy were observed. Due to lack of significant differentiation in the classification results, further analyses on these data were not considered.

3.4 Discussion

The analysis of the fungal communities associated with wheat roots, based on the ITS region sequencing, revealed that the phyla Ascomycota, Basidiomycota, Mortierellomycota, and Glomeromycota were dominant. This distribution of fungal phyla is consistent with other high-throughput sequencing studies of rhizospheric fungal communities in various plants, including wheat (Sommermann et al., 2018; Gqozo et al., 2020; Li et al., 2020).

The findings indicated that fungal abundance was significantly influenced by the inoculation treatment. Plants inoculated with soil containing AMF spores, and other rhizospheric microorganisms exhibited statistically different fungal abundances compared to non-inoculated wheat plants, which supports findings from previous studies showing that AMF inoculation enhances fungal colonisation and diversity (Li et al., 2020).

Additionally, inoculation led to increased ASV richness and diversity, which was observed to vary across the different wheat landraces. This suggests that wheat genotype played a crucial role in determining the composition and diversity of fungal communities in the root microbiome, aligning with findings by Campos et al. (2018) that reported genotype-dependent variations in fungal colonisation.

Interestingly, drought stress did not significantly impact fungal community composition or diversity in the majority of treatments. This result is consistent with the study by Kozjek et al. (2021), which found that short-term drought stress did not alter the diversity of fungal and bacterial communities in field conditions, with microbial communities being more responsive to other environmental and management-related variables. However, in contrast to the general trend, a significant difference in the relative abundance was observed in the inoculated landrace C3 under drought stress, where fungal abundance was notably higher compared to its non-inoculated control and the well-watered controls.

The overall fungal abundance and community composition were primarily influenced by the inoculation treatment and wheat genotype, rather than by drought stress. These findings could further be supported by the microscopically determined percentage of root colonisation by AMF and non-AMF endophytes presented in Chapter 2.

The correlation between the inoculation treatment and fungal community dynamics underlines the importance of fungal inoculants in modulating the wheat microbiome, particularly in stress environments, as corroborated by previous research on AMF colonisation and plant-fungal interactions under drought conditions (Salamon et al., 2020).

On the other hand, the analysis of the 18S rRNA gene sequencing did not successfully produce a taxonomical classification of AMF, despite attempts to adjust variables at various stages of the DADA2 pipeline. Thus, a more likely explanation of this limitation could be attributed to potential inconsistencies in DNA sample quality, which may had been influenced by drought, inoculation treatments (Yang et al., 2005), or to DNA extraction and protocols employed, since primers have failed to efficiently separate the wheat DNA from the fungal DNA, based on the high level of matching against the IWGSC in BLAST analysis.

Despite some limitations encountered, inoculation and drought effects and differences on wheat landraces were clear. However, the low percentage of AMF root colonisation, as reported in Chapter 2, suggested that the times and/or sample collection were not adequate, thus a better experimental design is required to understand the underlying mechanisms governing these interactions.

Therefore, while the research succeeded in meeting its main objectives of understanding the fungal community composition within wheat landraces (with some limitations), the impact of drought stress on fungal community dynamics had not been fully documented in this experimental research. Thus, exploring different approaches especially over short or long term, or under more moderate drought conditions, different AMF communities or more diverse wheat landraces panels, are

necessary to clarify the complex relationships between fungal communities, drought stress, and wheat landraces.

3.5 Appendix S3

Table S3.1 Pipeline of the QIIME2 (Quantitative Insights Into Microbial Ecology) script used for the analysis of the sequencing reads from the ITS region and SSU rRNA gene. The pipeline was based on (https://forum.qiime2.org/t/fungal-its-analysis-tutorial/7351).

1. Fit a classifier for the UNITE
sh_refs_qiime_ver9_**97**_16.10.2022.fasta sh_refs_qiime_ver9_**99**_16.10.2022.fasta sh_refs_qiime_ver9_dynamic_16.10.2022.fasta sh_taxonomy_qiime_ver9_**97**_16.10.2022.txt sh_taxonomy_qiime_ver9_**99**_16.10.2022.txt sh_taxonomy_qiime_ver9_dynamic_16.10.2022.txt #Folder developer: sh_refs_qiime_ver9_**97**_16.10.2022_**dev**.fasta sh_refs_qiime_ver9_**99**_16.10.2022_**dev**.fasta sh_refs_qiime_ver9_dynamic_16.10.2022_**dev**.fasta sh_taxonomy_qiime_ver9_**97**_16.10.2022_**dev**.txt sh_taxonomy_qiime_ver9_**99**_16.10.2022_**dev**.txt sh_taxonomy_qiime_ver9_dynamic_16.10.2022_**dev**.txt
2. Scrub lowercase characters from the sequences to avoid errors downstream
awk '/^>/ {print($0)}; /^[^>]/ {print(toupper($0))}' developer/sh_refs_qiime_ver9_99_16.10.2022_**dev**.fasta | tr -d ' ' > developer/sh_refs_qiime_ver9_99_16.10.2022_**dev**_uppercase.fasta qiime tools import \\ --type FeatureData[**Sequence**] \\ --input-path developer/sh_refs_qiime_ver9_99_16.10.2022_**dev_uppercase**.fasta \\ --output-path unite-ver9-**99-seqs**-16.10.2022.**qza** qiime tools import \\ --type FeatureData[**Taxonomy**] \\ --input-path developer/sh_**taxonomy**_qiime_ver9_99_16.10.2022_**dev**.txt \\ --output-path unite-ver9-**99-tax**-16.10.2022.**qza** \\ --input-format HeaderlessTSVTaxonomyFormat
3. Train the classifier
qiime feature-**classifier** fit-**classifier-naive-bayes** \\ --i-reference-**reads** unite-ver9-**99-seqs**-16.10.2022.qza \\ --i-reference-**taxonomy** unite-ver9-**99-tax**-16.10.2022.qza \\ --o-classifier unite-ver9-**99-classifier**-16.10.2022.**qza**
4. Your Data
#Copy your sequencing files to the folder in the working directory
5. Create a fastq manifest file for importing data into QIIME2
#(File saved as **fastqmanifest.csv**) qiime tools import \\ --type SampleData[PairedEndSequencesWithQuality] \\ --input-path **fastqmanifest.csv** \\ --output-path demux.qza \\ --input-format PairedEndFastqManifestPhred33 qiime demux summarize \\ --i-data demux.qza \\ --o-visualization demux.**qzv**

qiime tools view demux.**qzv**

6. Trim Primers (gITS7-ITS4)

qiime cutadapt **trim-paired** \
--i-demultiplexed-sequences demux.qza \
--p-adapter-f GCATATCAATAAGCGGAGGA \ #→ **(R) rc**
--p-front-f GTGARTCATCGARTCTTTG\ #→ **(F) gITS7**
--p-adapter-r CAAAGAYTCGATGAYTCAC\ #→ **(F) rc**
--p-front-r TCCTCCGCTTATTGATATGC \ #→ **(R) ITS4**
--o-trimmed-sequences demux-trimmed.qza

qiime demux summarize \
--i-data demux-trimmed.qza \
--o-visualization demux-trimmed.**qzv**

7. Denoising → Amplicon Sequence Variants (ASVs)

Depending on the read quality use: denoise-**paired** or denoise-**single** (using only the forward reads)

qiime dada2 denoise-**single** \
--i-demultiplexed-seqs demux-trimmed.**qza** \
--p-trim-left 230 \
--p-trunc-len 150 \
--o-representative-sequences dada2-single-end-rep-seqs.qza \
--o-table dada2-**single**-end-table.qza \
--o-denoising-stats dada2-**single**-end-stats.qza

qiime metadata tabulate \
--m-input-file dada2-**single**-end-stats.qza \
--o-visualization dada2-**single**-end-stats.qzv

8. Taxonomic classification with UNITE

qiime feature-classifier classify-**sklearn** \
--i-classifier unite-ver9-99-classifier-16.10.2022.qza \
--i-reads dada2-**single**-end-rep-seqs.qza \
--o-classification taxonomy- **single**-end.qza

qiime taxa barplot \
--i-table dada2-**single**-end-table.qza \
--i-taxonomy taxonomy-**single**-end.qza \
--m-metadata-file **metadata.txt** \
--o-visualization taxa-bar-plots.qzv

#To generate a Tree
qiime phylogeny align-to-tree-mafft-fasttree \
--i-sequences dada2-**single**-end-rep-seqs.qza \
--o-alignment dada2-aligned-rep-seqs.qza \
--o-masked-alignment masked-aligned-rep-seqs.qza \
--o-tree fasttree-unrooted-tree.qza \
--o-rooted-tree fasttree-rooted-tree.qza \
--verbose

Table S3.2 Pipeline of the DADA2 (Divisive Amplicon Denoising Algorithm) used for the analysis of the sequencing reads from the 18S rRNA gene. The pipeline was based on (Caporaso et al. (2011); https://benjjneb.github.io/dada2/ITS_workflow.html).

1. Starting point

```
path <- "~/test/ITS_tutorial"  ## CHANGE ME to the directory containing the fastq files.
list.files(path)

fnFs <- sort(list.files(path, pattern = "_1.fastq.gz", full.names = TRUE))
fnRs <- sort(list.files(path, pattern = "_2.fastq.gz", full.names = TRUE))
```

2. Identify primers

```
FWD <- "ACCTGCGGARGGATCA"  ## CHANGE ME to your forward primer sequence
REV <- "GAGATCCRTTGYTRAAAGTT"  ## CHANGE ME...

allOrients <- function(primer) {
  # Create all orientations of the input sequence
  require(Biostrings)
  dna <- DNAString(primer)  # The Biostrings works w/ DNAString objects rather than character
vectors
  orients <- c(Forward = dna, Complement = Biostrings::complement(dna), Reverse =
Biostrings::reverse(dna),
      RevComp = Biostrings::reverseComplement(dna))
  return(sapply(orients, toString))  # Convert back to character vector
}
FWD.orients <- allOrients(FWD)
REV.orients <- allOrients(REV)
FWD.orients
```

```
fnFs.filtN <- file.path(path, "filtN", basename(fnFs)) # Put N-filtered files in filtN/ subdirectory
fnRs.filtN <- file.path(path, "filtN", basename(fnRs))

filterAndTrim(fnFs, fnFs.filtN, fnRs, fnRs.filtN, maxN = 0, multithread = TRUE)
primerHits <- function(primer, fn) {
  # Counts number of reads in which the primer is found
  nhits <- vcountPattern(primer, sread(readFastq(fn)), fixed = FALSE)
  return(sum(nhits > 0))
}
rbind(FWD.ForwardReads = sapply(FWD.orients, primerHits, fn = fnFs.filtN[[1]]),
FWD.ReverseReads = sapply(FWD.orients,
  primerHits, fn = fnRs.filtN[[1]]), REV.ForwardReads = sapply(REV.orients, primerHits,
  fn = fnFs.filtN[[1]]), REV.ReverseReads = sapply(REV.orients, primerHits, fn = fnRs.filtN[[1]]))
```

3. Remove Primers

```
cutadapt <- "/usr/local/bin/cutadapt" # CHANGE ME to the cutadapt path on your machine
system2(cutadapt, args = "--version") # Run shell commands from R
```

```
path.cut <- file.path(path, "cutadapt")
if(!dir.exists(path.cut)) dir.create(path.cut)
fnFs.cut <- file.path(path.cut, basename(fnFs))
fnRs.cut <- file.path(path.cut, basename(fnRs))

FWD.RC <- dada2:::rc(FWD)
REV.RC <- dada2:::rc(REV)
# Trim FWD and the reverse-complement of REV off of R1 (forward reads)
R1.flags <- paste("-g", FWD, "-a", REV.RC)
# Trim REV and the reverse-complement of FWD off of R2 (reverse reads)
R2.flags <- paste("-G", REV, "-A", FWD.RC)
# Run Cutadapt
for(i in seq_along(fnFs)) {
 system2(cutadapt, args = c(R1.flags, R2.flags, "-n", 2, # -n 2 required to remove FWD and REV
from reads
                  "-o", fnFs.cut[i], "-p", fnRs.cut[i], # output files
```

```
                fnFs.filtN[i], fnRs.filtN[i])) # input files
}
rbind(FWD.ForwardReads = sapply(FWD.orients, primerHits, fn = fnFs.cut[[1]]),
FWD.ReverseReads = sapply(FWD.orients,
  primerHits, fn = fnRs.cut[[1]]), REV.ForwardReads = sapply(REV.orients, primerHits,
  fn = fnFs.cut[[1]]), REV.ReverseReads = sapply(REV.orients, primerHits, fn = fnRs.cut[[1]]))
# Forward and reverse fastq filenames have the format:
cutFs <- sort(list.files(path.cut, pattern = "_1.fastq.gz", full.names = TRUE))
cutRs <- sort(list.files(path.cut, pattern = "_2.fastq.gz", full.names = TRUE))

# Extract sample names, assuming filenames have format:
get.sample.name <- function(fname) strsplit(basename(fname), "_")[[1]][1]
sample.names <- unname(sapply(cutFs, get.sample.name))
head(sample.names)
```

4. Inspect read quality profiles

```
plotQualityProfile(cutFs[1:2])
plotQualityProfile(cutRs[1:2])
```

5. Filter and trim

```
filtFs <- file.path(path.cut, "filtered", basename(cutFs))
filtRs <- file.path(path.cut, "filtered", basename(cutRs))
out <- filterAndTrim(cutFs, filtFs, cutRs, filtRs, maxN = 0, maxEE = c(2, 2), truncQ = 2,
  minLen = 50, rm.phix = TRUE, compress = TRUE, multithread = TRUE)  # on windows, set
multithread = FALSE
head(out)
```

6. Learn the Error Rates

```
errF <- learnErrors(filtFs, multithread = TRUE)
errR <- learnErrors(filtRs, multithread = TRUE)
# Visualize the estimated error rates as a sanity check.
plotErrors(errF, nominalQ = TRUE)
```

7. Sample Inference

```
dadaFs <- dada(filtFs, err = errF, multithread = TRUE)
dadaRs <- dada(filtRs, err = errR, multithread = TRUE)
```

8. Merge paired reads

```
mergers <- mergePairs(dadaFs, filtFs, dadaRs, filtRs, verbose=TRUE)
```

9. Construct Sequence Table

```
seqtab <- makeSequenceTable(mergers)
dim(seqtab)
```

10. Remove chimeras

```
seqtab.nochim <- removeBimeraDenovo(seqtab, method="consensus", multithread=TRUE,
verbose=TRUE)
table(nchar(getSequences(seqtab.nochim)))
```

11. Track reads through the pipeline

```
getN <- function(x) sum(getUniques(x))
track <- cbind(out, sapply(dadaFs, getN), sapply(dadaRs, getN), sapply(mergers, getN),
  rowSums(seqtab.nochim))
# If processing a single sample, remove the sapply calls: e.g. replace
# sapply(dadaFs, getN) with getN(dadaFs)
colnames(track) <- c("input", "filtered", "denoisedF", "denoisedR", "merged", "nonchim")
rownames(track) <- sample.names
head(track)
```

12. Assign taxonomy

```
unite.ref <- "~/tax/sh_general_release_dynamic_s_all_29.11.2022.fasta"  # CHANGE ME to
location on your machine
taxa <- assignTaxonomy(seqtab.nochim, unite.ref, multithread = TRUE, tryRC = TRUE)
taxa.print <- taxa  # Removing sequence rownames for display only
rownames(taxa.print) <- NULL
head(taxa.print)
```

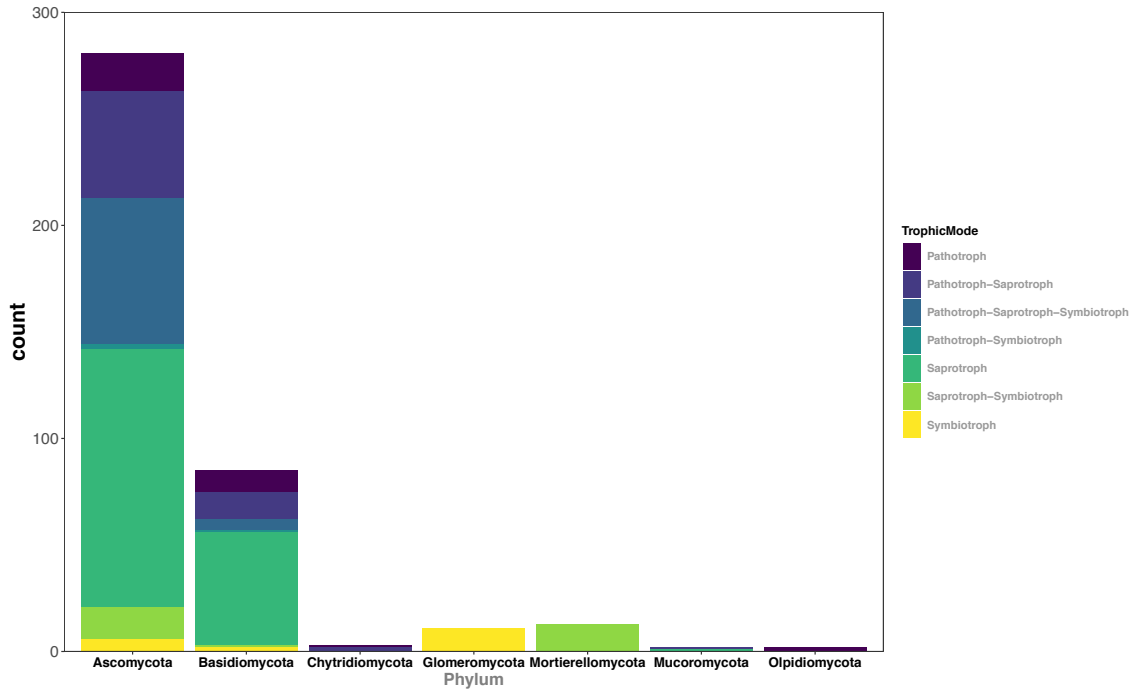


Figure S3.1 | Counts of fungal sequences represented by the trophic mode (using FUNGuild) on the phyla (Ascomycota, Basidiomycota, Mortierellomycota, Glomeromycota, Chytridiomycota, Mucoromycota and Olpidiomycota) found in the different wheat samples.

Chapter 4. Investigating the effect of inoculation with *Rhizophagus irregularis* BEG72 on spring wheat landraces under two contrasting water regimes

4.1 Introduction

AMF play a crucial role in plant nutrition and stress tolerance by enhancing nutrient uptake, particularly phosphorus, and improving water relations (Smith and Read, 2008). Among the various AMF species, *Rhizophagus irregularis* has emerged as a model organism due to its global distribution and ability to colonise a wide range of host plants (Öpik et al., 2013).

In recent years, there has been growing interest in utilising AMF as biofertilizers to enhance crop productivity and sustainability in agricultural systems. Inoculation with *R. irregularis* has shown promise in improving plant growth, yield, and nutrient uptake in various crops, including wheat (Pellegrino et al., 2015). However, the effectiveness of AMF inoculation can vary depending on numerous factors, including host plant genotype, environmental conditions, and indigenous AMF communities (Sawers et al., 2017).

Water availability is a critical factor influencing plant growth and crop productivity, particularly in the face of climate change and increasing water scarcity. Drought stress can significantly impact plant physiology, metabolism, and yield (Farooq et al., 2009). AMF colonisation has been shown to enhance drought tolerance in various plant species by improving water uptake, osmotic adjustment, and antioxidant defence systems (Augé et al., 2015). However, the interaction between AMF inoculation and water availability on plant performance remains complex and requires further investigation.

On the other hand, wheat landraces, which are locally adapted varieties traditionally cultivated by farmers, represent an important genetic resource for breeding

programs aimed at developing resilient and high-yielding cultivars. These landraces often possess valuable traits such as adaptability to local environmental conditions and resistance to biotic and abiotic stresses (Lopes et al., 2015). Exploring the response of spring wheat landraces to AMF inoculation under different water regimes could provide valuable insights into their potential for sustainable agriculture and breeding programs.

The AMF species *R. irregularis* has been widely studied and used in inoculation experiments due to its broad host range and efficient colonisation capabilities (Köhl et al., 2016). However, the specific effects of this strain on spring wheat landraces under varying water conditions have not been thoroughly investigated. Understanding the interactions between *R. irregularis*, wheat landraces, and water availability (drought stress) could contribute to the development of more effective inoculation strategies and the selection of wheat genotypes better suited for symbiotic associations.

4.1.1. Aims and objectives

The objectives of this experimental project were: (a) To assess the effects of drought stress on 30 spring wheat landraces inoculated with the arbuscular mycorrhizal fungus *Rhizophagus irregularis* BEG72. This was achieved by evaluating various physiological and morphological parameters, including total chlorophyll content at different developmental stages, dry biomass (above and below-ground), plant height, spike length, number of grains per spike, total grain number per plant, and overall grain yield per plant. It was hypothesised that inoculation of wheat landraces with commercial inoculum *R. irregularis* BEG72 will mitigate the negative effects of drought stress on plants, resulting in improved growth and yield-related traits in the inoculated wheat landraces compared to non-inoculated controls.

(b) To quantify the percentage of AMF colonisation using relative quantification via qPCR, and to compare these results with the colonisation percentage obtained through microscopic analysis. For this objective it was hypothesised that the relative qPCR quantification of AMF colonisation will correlate positively with microscopy-

based percentage of colonisation, providing a reliable molecular alternative for determining AMF presence in wheat roots.

4.2 Materials and methods

4.2.1. Plant material: wheat diversity panel

A collection of *T. aestivum* landraces from the YoGI biodiversity panel (Barratt et al., 2023) were selected (Table 4.1). A name consisting of one letter and two numbers was given to each landrace. The MEs for these landraces were omitted since they could not be precisely established.

Table 4.1 Spring wheat landraces from the YoGI collection used in this research experiment. The assigned name and area of origin are also indicated.

LANDRACE		NAME	ORIGIN
1.	YoGI_036	**A36**	Argentina
2.	YoGI_037	**A37**	Australia
3.	YoGI_040	**A40**	Australia
4.	YoGI_032	**B32**	Brazil
5.	YoGI_145	**C45**	China
6.	YoGI_050	**C50**	China
7.	YoGI_057	**C57**	Canada
8.	YoGI_258	**C58**	China
9.	YoGI_063	**E63**	Ecuador
10.	YoGI_117	**F17**	France
11.	YoGI_109	**I09**	Italy
12.	YoGI_228	**I12**	Iran
13.	YoGI_028	**I28**	India
14.	YoGI_261	**I61**	Iran
15.	YoGI_088	**I88**	India
16.	YoGI_047	**J47**	Japan
17.	YoGI_009	**K09**	Kenya
18.	YoGI_312	**K12**	Kazakhstan
19.	YoGI_309	**M09**	Mongolia
20.	YoGI_119	**M19**	Morocco
21.	YoGI_073	**M73**	Mexico
22.	YoGI_006	**P06**	Pakistan
23.	YoGI_070	**R70**	Russian Fed.
24.	YoGI_016	**S16**	South Africa
25.	YoGI_019	**S19**	South Africa

26.	YoGI_320	**S20**	Saudi Arabia
27.	YoGI_061	**T61**	Türkiye
28.	YoGI_163	**T63**	Türkiye
29.	YoGI_043	**U43**	USA
30.	YoGI_293	**U93**	USSR

4.2.2. AMF inoculation and irrigation treatments

Wheat seeds were washed and germinated as described in Chapter 2 (Section 2.2 Materials and Methods). In this research experiment, seedlings were transferred to 1.5 L pots containing a 50:50 (v/v) mix of sand:Terra-Green®. For each wheat genotype, one seedling per pot was sown with five replicates per treatment (n=20), plus two sacrifice pots to observe the establishment of colonisation.

Wheat plants were grown for approximately 40-50 days in a greenhouse at temperatures ranging from 12.8 to 30 °C, with 10-14 hours of natural light and 14-10 hours of darkness. The pots were fertilised with a modified Hoagland nutrient solution (without N and P); instead, they received bonemeal (3.5% N and 8.7% P), as described by Leigh et al. (2011). The experimental design was a completely randomised block design to prevent environmental gradients that could influence plant development.

Twelve seedlings of each wheat landrace were inoculated with 20g per pot of a commercial inoculum consisting of spores and AMF-inoculated root pieces of *Rhizophagus irregularis* BEG72 (PlantWorks Limited, Kent). At the same time, ten wheat seedlings that did not receive the inoculum served as controls.

Two irrigation treatments were established based on field capacity: well-watered plants at 95% FC and drought-stressed plants at 60% FC. During the first four weeks, all plants were watered to maintain 95% FC to enhance the chances of colonisation and establishment of the AMF. Plants under drought stress treatment were weighed every two days, and water was added to replace the loss via evapotranspiration to maintain their original weight.

4.2.3. Preliminarily detection of root colonisation by AMF

To ensure that the wheat landraces were successfully colonised by the AMF from the commercial inoculum, two sacrifice pots per landrace were grown alongside the main experimental pots, following the same procedures. One plant from each sacrifice pot was harvested three weeks after sowing, and the other at four weeks, to collect root samples and observe their colonisation status under the microscope.

Positive colonisation was confirmed when the AMF formed arbuscules inside the wheat roots. This colonisation predominantly occurred by the third week after sowing, with completion by the fourth week in all wheat landraces. Once colonisation was confirmed, the plants were subjected to various treatments.

4.2.4. Chlorophyll measurement

Total chlorophyll was measured and recorded at 21, 28, 35, 42, 49, and 56 days after sowing, corresponding to the Zadoks growth scale from SG20 to SG60-69 (Zadoks et al., 1974). The atLEAF CHL values were converted to total chlorophyll content, expressed in milligrams per square centimetre (mg/cm^2), using the conversion tool available on the website of the manufacturer (https://www.atleaf.com).

4.2.5. Harvesting, biomass and grain production

Harvesting of dry biomass and grain yield was carried out as described in Chapter 2 (Section 2.2 Materials and Methods).

4.2.6. Percentage of AMF root colonisation by microscopy and qPCR (relative quantification)

A freeze-dried root sample from each wheat plant was taken and cut into 1 cm pieces for microscopic examination of AMF structures, including arbuscules, hyphae, spores, and vesicles. The root samples were prepared for staining as described in

Chapter 2 (Section 2.2 Materials and Methods), and the percentage of colonisation was determined using the magnified intersections method (McGonigle et al., 1990).

Relative qPCR quantification of AMF root colonisation was conducted following the approach described by Bodenhausen et al. (2021), which demonstrated a strong correlation between AMF root colonisation detected by microscopy and relative qPCR results. The study employed a set of primers, AMG1F-AM1, that bind to the 18S rRNA gene of the small subunit ribosomal operon, recognising various AMF species, including *R. irregularis*. As an internal standard in qPCR for wheat, specific primers to an actin gene, a common housekeeping gene in various plant species, were used (Table 4.2). This pair of primers (TaActin) were previously used as an internal standard in quantitative PCR for wheat under drought stress by Mega et al. (2019).

Table 4.2 Primer pairs used for the qPCR assay, the first pair recognises a conserved segment in arbuscular mycorrhizal fungi, and the second pair recognises a housekeeping plant gene.

PRIMER	SEQUENCE (5' - 3')	SIZE (BP)	GENE	REFERENCE
AMG1F (F)	ATA GGG ATA GTT GGG GGC AT	230	18S rRNA (AMF)	Hewins et al., 2015
AM1 (R)	GTT TCC CGT AAG GCG CCG AA			Helgason et al., 1998
TaActin (F)	CCT CTC TGC GCC AAT CGT	156	Actin (wheat)	Mega et al., 2019
TaActin (R)	TCA GCC GAG CGG GAA ATT GT			

Forward primer, (F); reverse primer (R). Ribosomal RNA (rRNA).

Both primer sets were verified for specificity using primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast). The AMG1F-AM1 primers matched the Glomeromycotina subphylum and showed no matches with other fungal or plant databases, including Ascomycota and *T. aestivum*. The TaActin primers specifically matched only the wheat database and showed no cross-reactivity with fungal databases, making them suitable for the qPCR analysis.

The qPCR assay was performed using a QuantStudio3™ (Applied Biosystems) with a 20 µL reaction mixture containing Fast SYBR™ Green Master Mix (Applied Biosystems), 250 nM of each primer, and approximately 10 ng of root DNA. The PCR programme included an initial denaturation at 95 °C for 20 seconds, followed by 40

cycles of denaturation at 95 °C for 1 second and an annealing step at 60 °C for 20 seconds. A melting curve was generated by increasing the temperature from 60 to 95 °C in 0.1 °C increments, holding for 5 seconds at each step. Each primer pair was tested in triplicate, and no-template controls were included in each run.

Data was exported from the Thermo Fisher Connect™ platform to Microsoft Excel for analysis. The average cycle number to threshold (Ct) of samples and primer efficiency were calculated as described by Bodenhausen et al. (2014; 2021). With primer efficiency meeting the expected range (90 to 110%), relative quantification was performed by normalising the AMF gene signal (18S rRNA) to the plant housekeeping gene (TaActin) using the mathematical model by Pfaffl, (2001).

4.2.7. Statistical analyses

The obtained data were subjected to a three-way analysis of variance (ANOVA) to evaluate the effects of the treatments. If the ANOVA results were significant, multiple pairwise comparisons of the sample means were performed using Tukey's Honestly Significant Difference (HSD) test (p <0.05), conducted with RStudio software (v. 4.2.2).

4.3 Results

4.3.1. Growth and development of wheat in greenhouse conditions

Thirty spring wheat landraces were germinated, transplanted into pots, and maintained in a greenhouse under controlled conditions. Each wheat genotype was subjected to four treatments: two levels of inoculation and two levels of irrigation. Intrinsic morphological differences were observed among the landraces, including variations in organ and tissue sizes as well as plant stature.

Additionally, several other morphological and physiological changes were noted. Specifically, seven landraces did not transition to the anthesis and flowering stages, which are necessary for grain production (Table 4.3). Instead, these landraces remained in the vegetative stage.

Table 4.3 Spring wheat landraces that did not transition to flowering stage.

LANDRACE		NAME
1.	YoGI_145	**C45**
2.	YoGI_258	**C58**
3.	YoGI_261	**I61**
4.	YoGI_119	**M19**
5.	YoGI_016	**S16**
6.	YoGI_320	**S20**
7.	YoGI_293	**U93**

4.3.2. R. irregularis enhanced chlorophyll levels under drought stress

Measurement of physiological plant responses can elucidate the effects of different biotic and abiotic conditions. In this research experiment, total chlorophyll content was recorded from week 4 to week 9 of wheat development, corresponding to the tillering stage (SG20) through the beginning and end of the anthesis stage (SG60-69) of the plants.

The total chlorophyll content varied significantly among the wheat genotypes and their interactions (Table 4.4). Specifically, inoculation with *R. irregularis* BEG72 resulted in increased chlorophyll levels in 73.9% of wheat landraces under drought stress conditions. In comparison, only 43.4% of the wheat landraces under well-watered conditions exhibited similar increases (0).

Table 4.4 Significance levels (F values) of Landrace (LA), Inoculation (IN), Irrigation (IR) and their interactions on measured parameters on a three-way ANOVA analysis. Significance levels: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ns, non-significant effect.

VARIABLES	LA	IN	IR	LA:IN	LA:IR	IN:IR	LA:IN:IR
Chlorophyll	24.16^{***}	16.72^{***}	6.94^{**}	2.74^{***}	2.53^{***}	12.58^{***}	1.00^{ns}
Above-ground	131.71^{***}	215.27^{***}	121.07^{***}	4.47^{***}	5.77^{***}	0.68^{ns}	2.12^{***}
Below-ground	66.48^{***}	3.89^{*}	46.85^{***}	2.40^{***}	2.57^{***}	3.30^{ns}	1.34^{ns}
Height	87.77^{***}	9.37^{**}	51.68^{***}	4.25^{***}	5.65^{***}	1.40^{ns}	1.86^{*}
Grain yield	34.46^{***}	95.97^{***}	26.53^{***}	3.95^{***}	1.95^{**}	0.96^{ns}	1.74^{*}

Therefore, in most cases, inoculated plants under drought stress exhibited similar or slightly elevated chlorophyll levels compared to the control. Notably, certain landraces, such as C50, P06 and K12 demonstrated a marked increase in chlorophyll following inoculation, while others, such as M73, showed a reduction in chlorophyll content under the same treatment.

Overall, inoculation had a variable effect on chlorophyll content, with a more apparent impact under drought stress compared to well-watered conditions. While some landraces appeared to benefit from inoculation in terms of enhanced chlorophyll maintenance, particularly under drought stress, others showed little difference or even reduced chlorophyll levels.

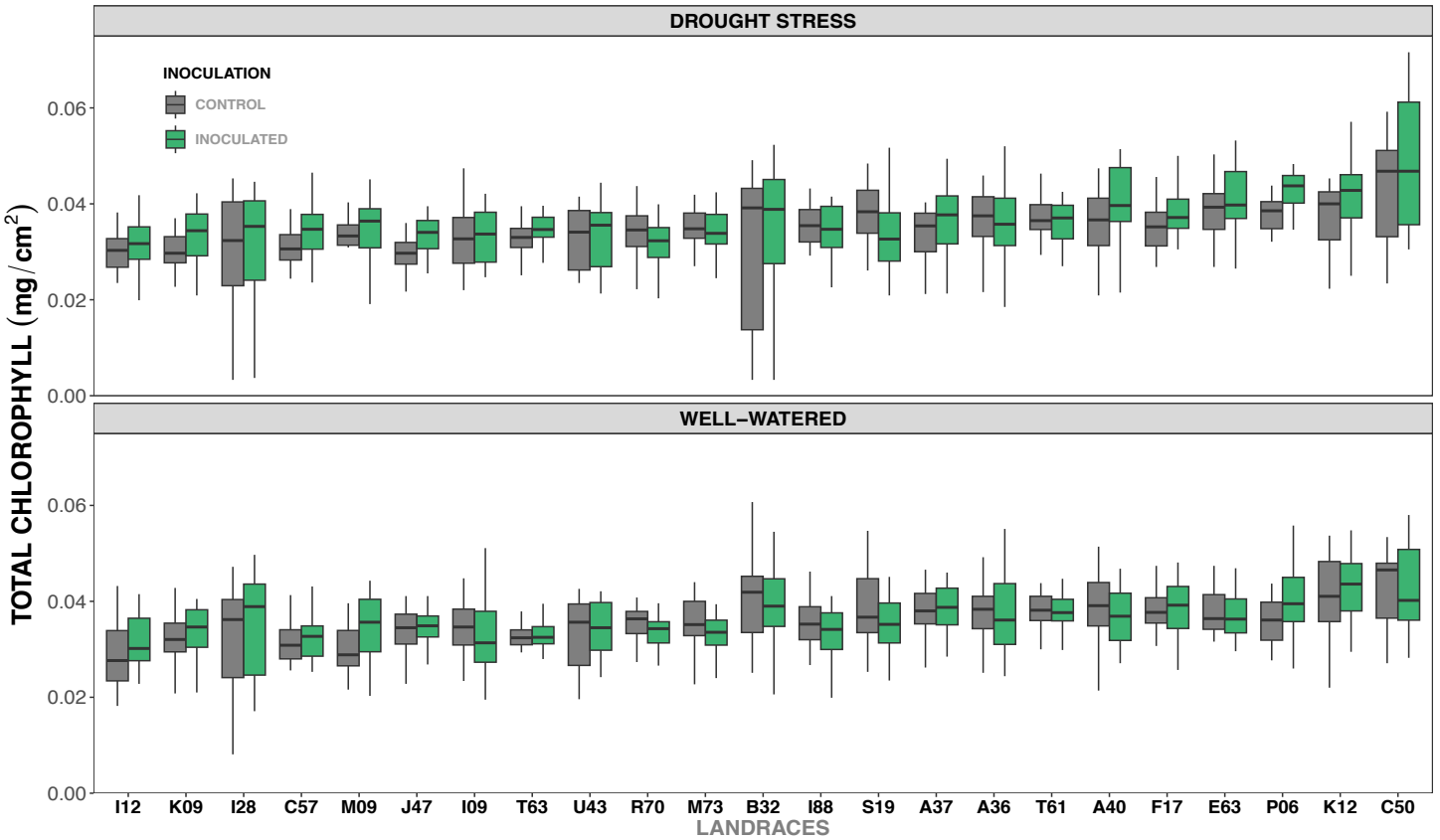


Figure 4.1 | Total chlorophyll (a and b) values (atLEAF) transformed to mg/cm² in 23 spring wheat landraces during six weeks (Zadoks' growth scale GS20 to 60-69) subjected to drought stress or under well-watered conditions at two inoculation levels: inoculated (AMF-inoculated) or control (non-inoculated). Every boxplot in each graph represents the mean of five replicates, thick lines represent medians, boxes indicate interquartile ranges, and whiskers show minimum and maximum chlorophyll values.

4.3.3. R. irregularis differentially influenced above-ground dry biomass under drought stress

With respect to above-ground biomass, the results revealed significant differences among wheat landraces, with inoculation and irrigation (drought stress) both influencing leaf and shoot dry biomass (Figure 4.3). Post hoc analysis (Table 4.4) confirmed that drought stress, regardless of inoculation status, significantly reduced aerial biomass in nearly all wheat genotypes when compared to their respective well-watered controls.

One of the most notable findings from these data is the strong positive influence of inoculation with *R. intraradices* on above-ground dry matter. The effect of inoculation was most pronounced in landraces such as T63, I88, and C50, where inoculated plants showed a significant increase in biomass compared to non-inoculated plants. However, in some landraces, such as P06 and M09, the biomass of inoculated plants remained comparable to that of control plants. Similar to drought stress, inoculated plants tended to have greater or equal biomass compared to control (non-inoculated) plants.

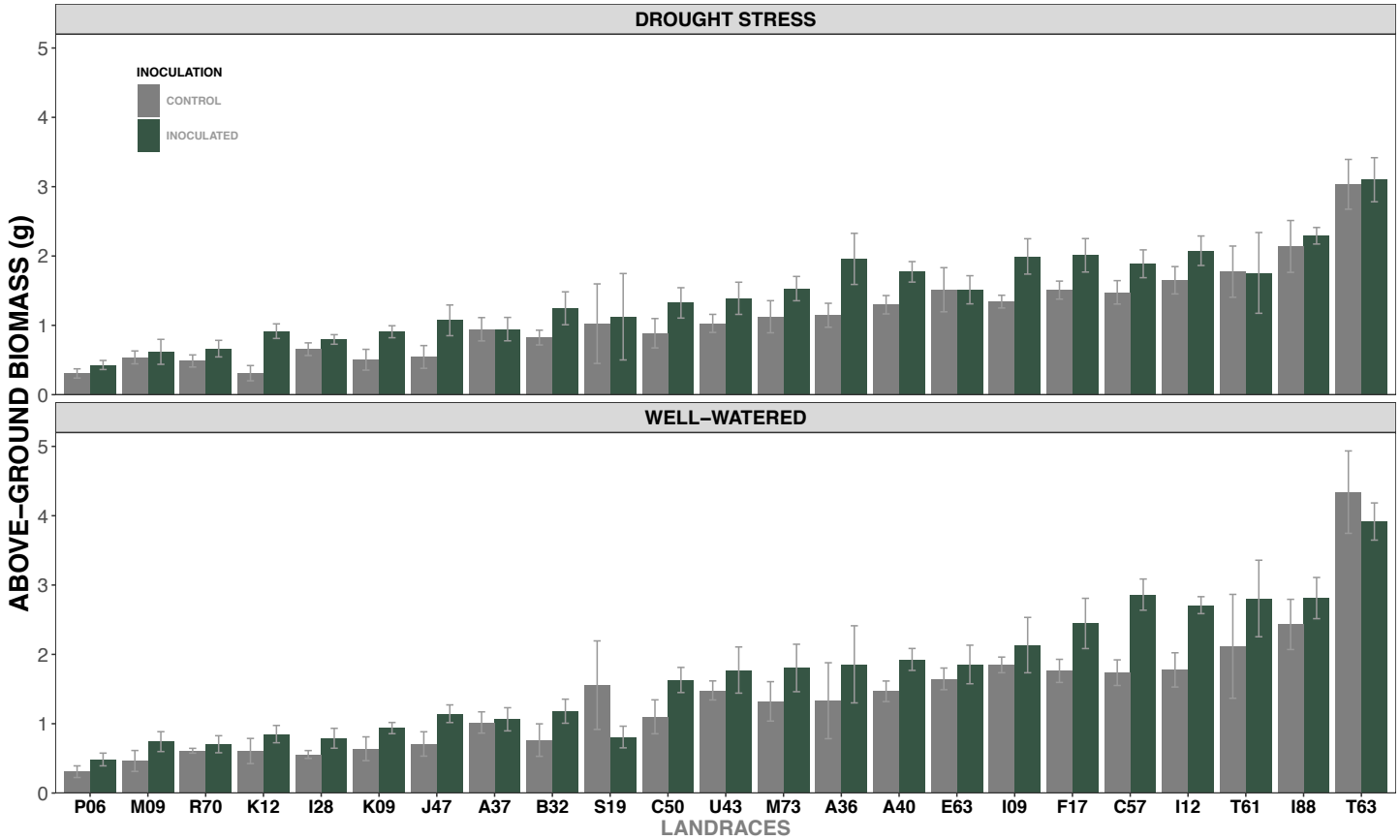


Figure 4.2 | Above-ground dry biomass (g) in 23 spring wheat landraces (means ± SD, n= 5) subjected to drought stress or under well-watered conditions at two inoculation levels: inoculated (AMF-inoculated) or control (non-inoculated). Significant differences among treatments were carried out by a three-way ANOVA followed by the Tukey's HSD test (p< 0.05).

4.3.4. R. irregularis differentially influenced below-ground dry biomass under drought stress

Regarding below-ground dry biomass, the response of the wheat landraces varied considerably (Figure 4.3). Drought stress proved to be a strong factor influencing root system dry weight, alongside inoculation and their interactions with individual landraces (Table 4.4). Despite the influence of these factors, there was no significant interaction between inoculation and irrigation.

Under drought stress, inoculated plants generally exhibited a higher below-ground biomass compared to the controls. Landraces such as K12, C50, A36 and A40 showed particularly strong responses to inoculation, with inoculated plants producing more root biomass than their control counterparts. However, various landraces, including I88, F17, and C57, displayed a reduction in root biomass in inoculated plants compared to the control. In the well-watered condition, inoculation led to increased root biomass in some landraces, particularly in C50, A40, I12, and J47, which also showed positive responses under drought stress.

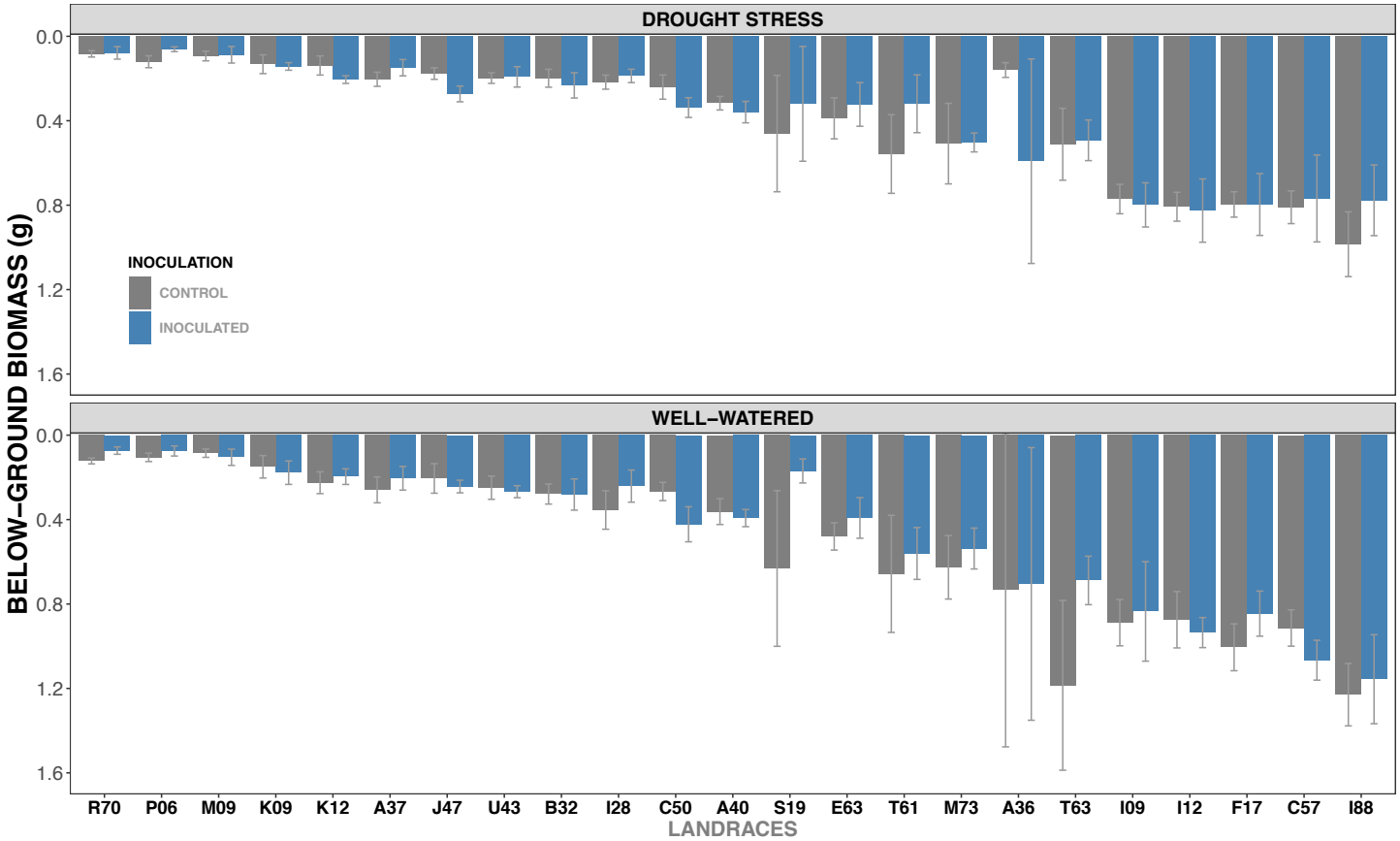


Figure 4.3 | Below-ground dry biomass (g) in 23 spring wheat landraces (means ± SD, *n*= 5) subjected to drought stress or under well-watered conditions at two inoculation levels: inoculated (AMF-inoculated) or control (non-inoculated). Significant differences among treatments were carried out by a three-way ANOVA followed by the Tukey's HSD test ($p < 0.05$).

4.3.5. Plant height

Plant height was observed to be significantly affected by drought stress (Figure 4.4). AMF inoculation, as well as its interaction with other variables, influenced the height of the different wheat landraces, although there was no significant interaction between inoculation and irrigation (Table 4.4).

Drought stress generally led to a reduction in plant height across nearly all landraces. Nevertheless, nine inoculated wheat landraces namely, A37, A40, C50, E63, F12, I12, I88, J47, and S19 exhibited greater height compared to their non-inoculated controls under drought conditions. Additionally, plant height exhibited a positive association with both above-ground biomass (R= 0.69, p< 0.001) and below-ground dry matter (R= 0.63, p< 0.001).

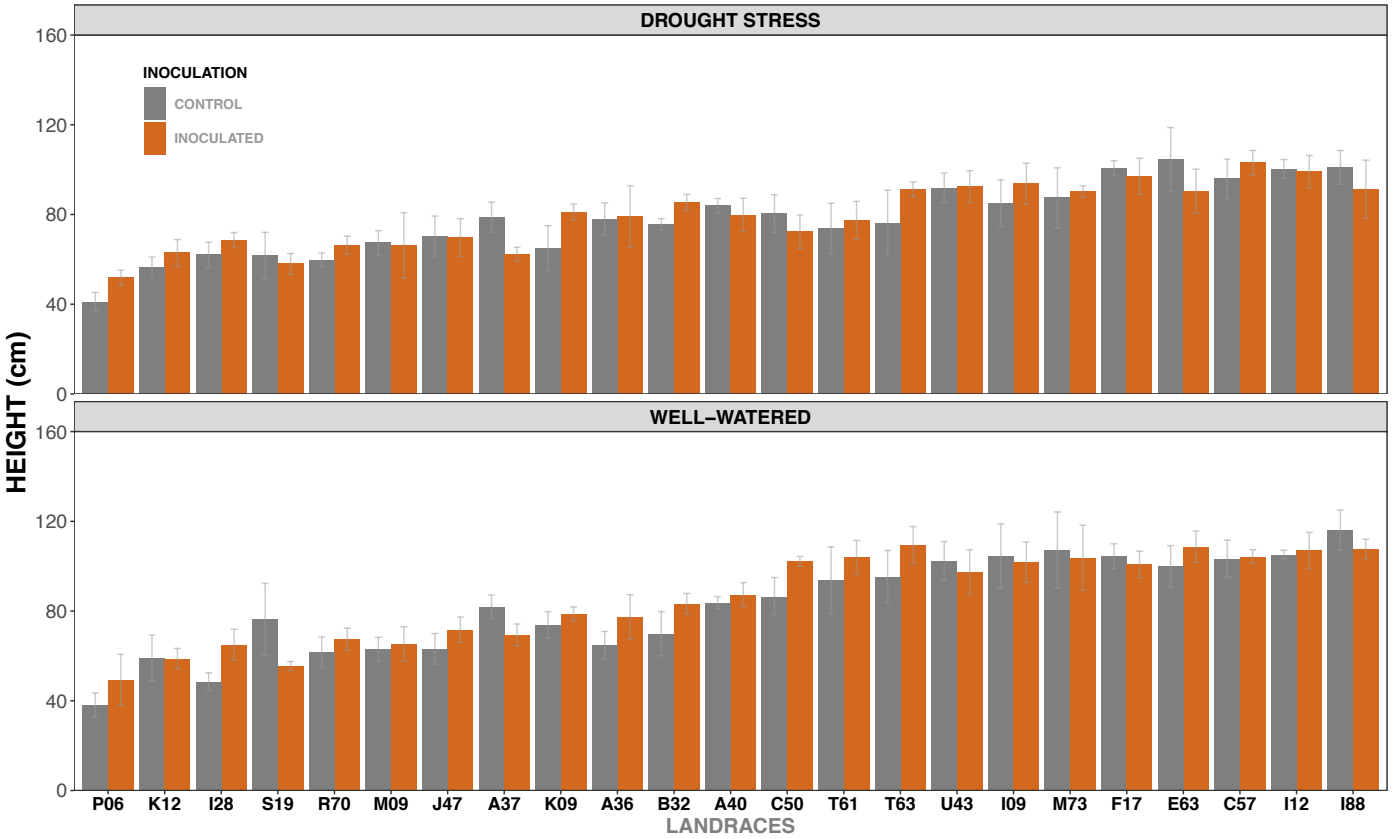


Figure 4.4 | Height (cm) of spring wheat landraces (means ± SD, *n*= 5) subjected to drought stress or under well-watered conditions at two inoculation levels: inoculated (AMF-inoculated) or control (non-inoculated). Significant differences among treatments were carried out by a three-way ANOVA followed by the Tukey's HSD test ($p< 0.05$).

4.3.6. Microscopic quantification of AMF root colonisation

Based on the above-ground and below-ground dry biomass results under drought stress, 12 spring wheat landraces were selected for detailed analysis: six with high total dry weight (A36, A40, F17, I09, I12, and T63) and six with low total dry biomass (I28, K09, K12, M09, P06, and R70). These landraces were assessed to quantify their root colonisation by AMF through microscopy and relative quantification qPCR.

Root samples from these 12 spring wheat landraces of each treatment were stained, and the percentage of colonisation was determined using the classical magnified intersections method (McGonigle et al., 1990). Figure 4.5 depicts the percentage of roots colonised by *R. irregularis* and the abundance of different fungal structures, including intraradical spores, vesicles, intraradical hyphae, and arbuscules. Control treatments (non-inoculated) were also analysed, but no AMF colonisation was observed. Non-AMF fungi were present in the roots of most landraces across both inoculation levels and irrigation treatments in a negligible percentage.

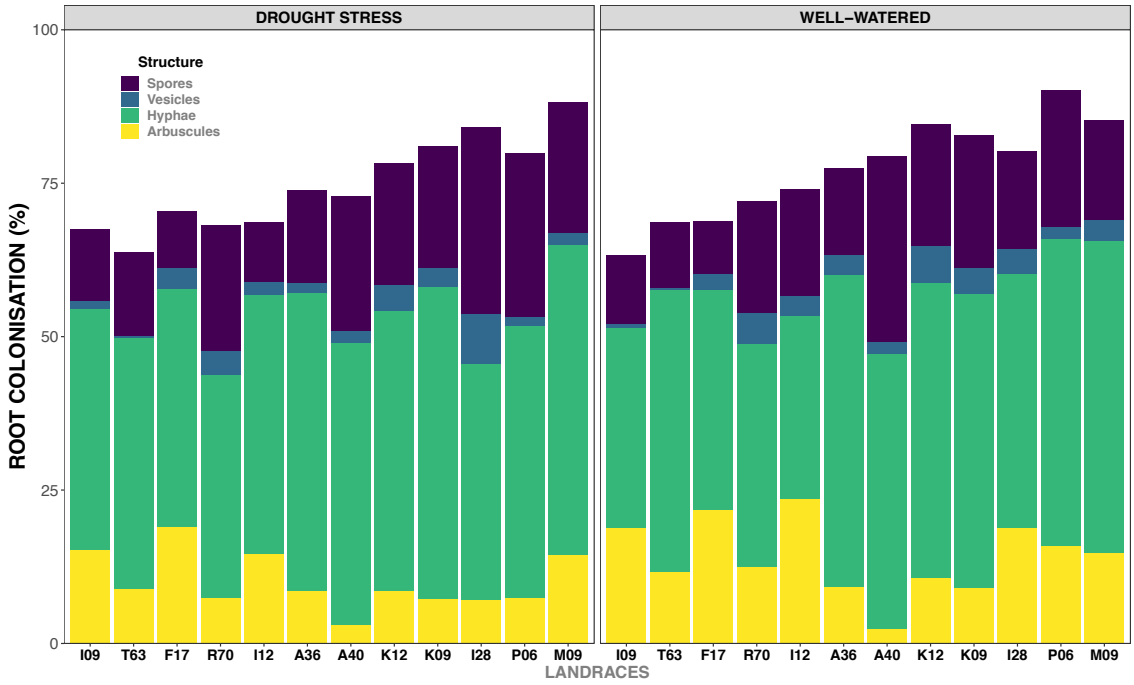


Figure 4.5 | Percentage of root colonisation by microscopy in 12 (inoculated) spring wheat landraces (means ± SD, *n*= 5) subjected to drought stress or under well-watered conditions. Fungal structures were recorded: intraradical spores, vesicles, intraradical hyphae and arbuscules. Significant differences among treatments were carried out by a three-way ANOVA followed by the Tukey's HSD test ($p< 0.05$).

Wheat genotype was a significant factor influencing the total percentage of root colonisation ($p< 0.001$). Drought stress generally did not affect AMF root colonisation, except for landraces P06 and T63, which showed significantly reduced colonisation under drought conditions. In contrast, landraces M09 and I09 performed significantly better under drought stress. The other landraces did not exhibit any notable differences compared to the well-watered control, except when specific fungal structures were examined.

Post hoc analysis showed that intraradical hyphae were the most prevalent AMF structures, while vesicles were the least abundant. However, landrace I28 and, to a lesser extent, P06 produced significantly more vesicles and intraradical spores under drought stress. In contrast, landraces A40 and I12 produced significantly more spores under well-irrigated conditions.

The most notable finding was the significant impact of drought stress on arbuscular abundance (p< 0.001) (Figure 4.6). Seventy-five per cent of the landraces exhibited a significantly lower percentage of arbuscules compared to non-stressed plants (p < 0.001), while the remaining landraces showed no significant changes in arbuscular abundance due to irrigation treatment.

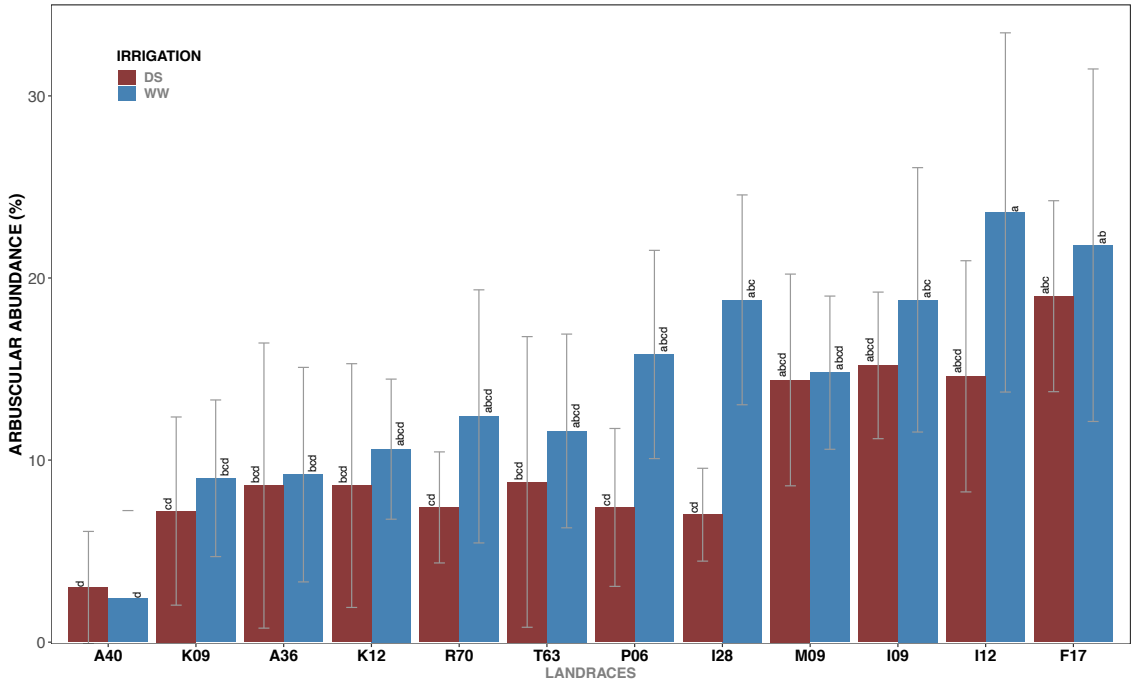


Figure 4.6 | Percentage of arbuscule abundance by microscopy in 12 (inoculated) spring wheat landraces (means ± SD, n= 5) subjected to drought stress (DS) or under well-watered (WW) conditions. Significant differences among treatments were carried out by a two-way ANOVA followed by the Tukey's HSD test (p< 0.05), columns followed by different letters are significantly different.

4.3.7. Relative quantification qPCR for AMF root colonisation quantification

The same 12 landraces used for microscopic analysis of root colonisation by *R. irregularis*, were also subjected to relative quantification analysis by qPCR. A standard curve was established using wheat DNA from a non-inoculated treatment, employing two sets of primers.

The cycle threshold (Ct) values for each primer pair across all dilutions were obtained (Figure S4.1). The slope was used to calculate primer efficiency, which was

found to be 93.8% for AMG1F-AM1 and 108.2% for TaActin, within the expected range. The primer efficiencies corresponded to values of 1.94 for AMG1F-AM1 and 2.08 for TaActin. Following the validation of primer efficiency, quantitative PCR was performed using root DNA from the wheat landraces. Figure 4.7 presents the log-transformed (log2) relative quantification of root colonisation by *R. irregularis* for each landrace, as determined by the qPCR method.

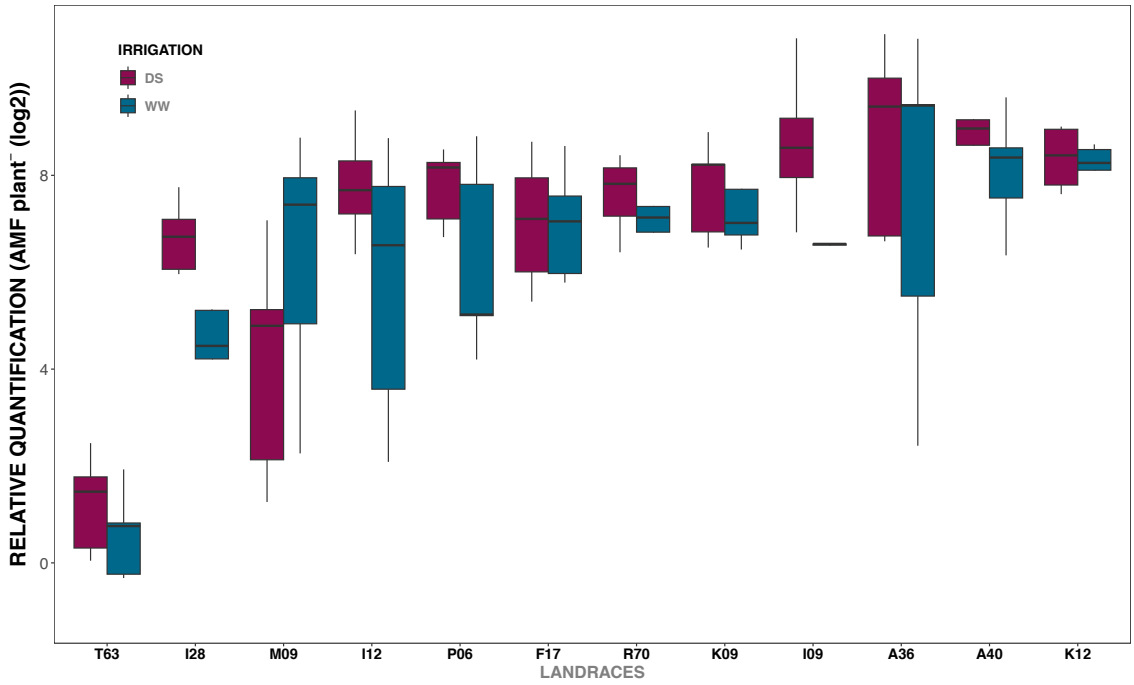


Figure 4.7 | Relative quantification calculated with the Pfaffl method of 12 inoculated spring wheat landraces subjected to drought stress (DS) or under well-watered (WW) conditions.

Relative AMF quantities varied significantly among wheat landraces ($p< 0.05$) with respect to the root colonisation by *R. irregularis*. Three drought-stressed wheat landraces A36, I28, and M09 demonstrated statistically significant differences compared to their well-irrigated counterparts ($p< 0.001$).

4.3.8. Microscopic versus relative quantification in qPCR of AMF root colonisation

The comparison between microscopy and qPCR methods for calculating the root AMF colonisation is shown in Figure 4.8. The sample-to-sample comparison

revealed that 71% of wheat landraces exhibited consistent root colonisation estimates across the two methods. Although a slight positive trend was observed when comparing the results, these findings should be interpreted with caution (R= 0.063, p= 0.49).

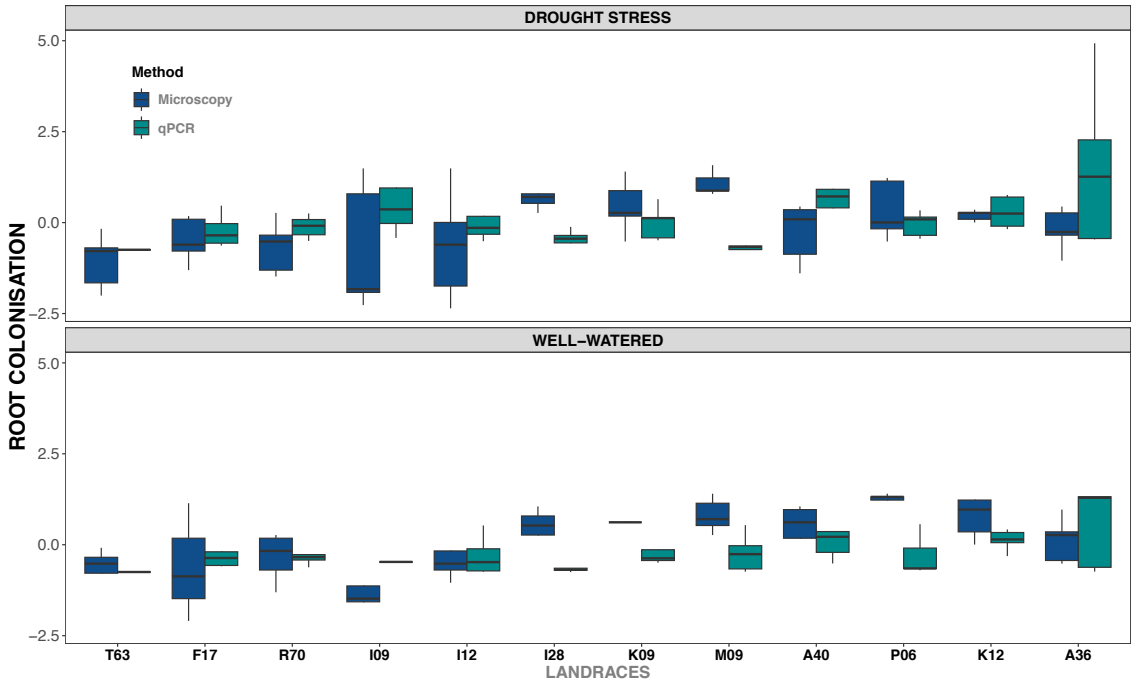


Figure 4.8 | Comparison of two quantification methods: microscopy and quantitative PCR, on normalised root colonisation data from 12 spring wheat landraces. Every boxplot in each graph represents the mean of five replicates, horizontal lines represent medians, boxes indicate interquartile ranges, and whiskers show minimum and maximum values.

Under drought stress, root colonisation levels were largely consistent between the two methods for most landraces. However, for certain landraces such as A36, qPCR method detected significantly higher colonisation compared to microscopy. In contrast, landraces such as T63, F17, and R70 displayed minimal variation between the two methods, indicating a closer alignment in colonisation detection under stress conditions.

Moreover, under well-watered conditions, greater variability was observed between the methods. For some landraces, such as P06 and T63, qPCR recorded significantly lower colonisation than microscopy. Conversely, in landraces like I09 and A36, qPCR identified higher colonisation levels than microscopy.

4.3.9. Grain production

Wheat grain production was assessed by collecting and weighing the harvested grains. Under well-watered conditions, the majority (91%) of wheat landraces inoculated with *R. intraradices* exhibited a significant increase in grain yield compared to their well-watered control counterparts (Table 4.4). Inoculated landraces such as A36, A37, and A40 showed a marked improvement in yield.

Under drought stress, inoculated plants generally produced higher grain yields compared to non-inoculated controls across most landraces (Figure 4.9). Notably, landraces such as A36 and A40 exhibited a substantial increase in yield compared with their controls. However, landraces such as I88, M09, and T61 showed minimal or no significant differences between inoculated and control plants.

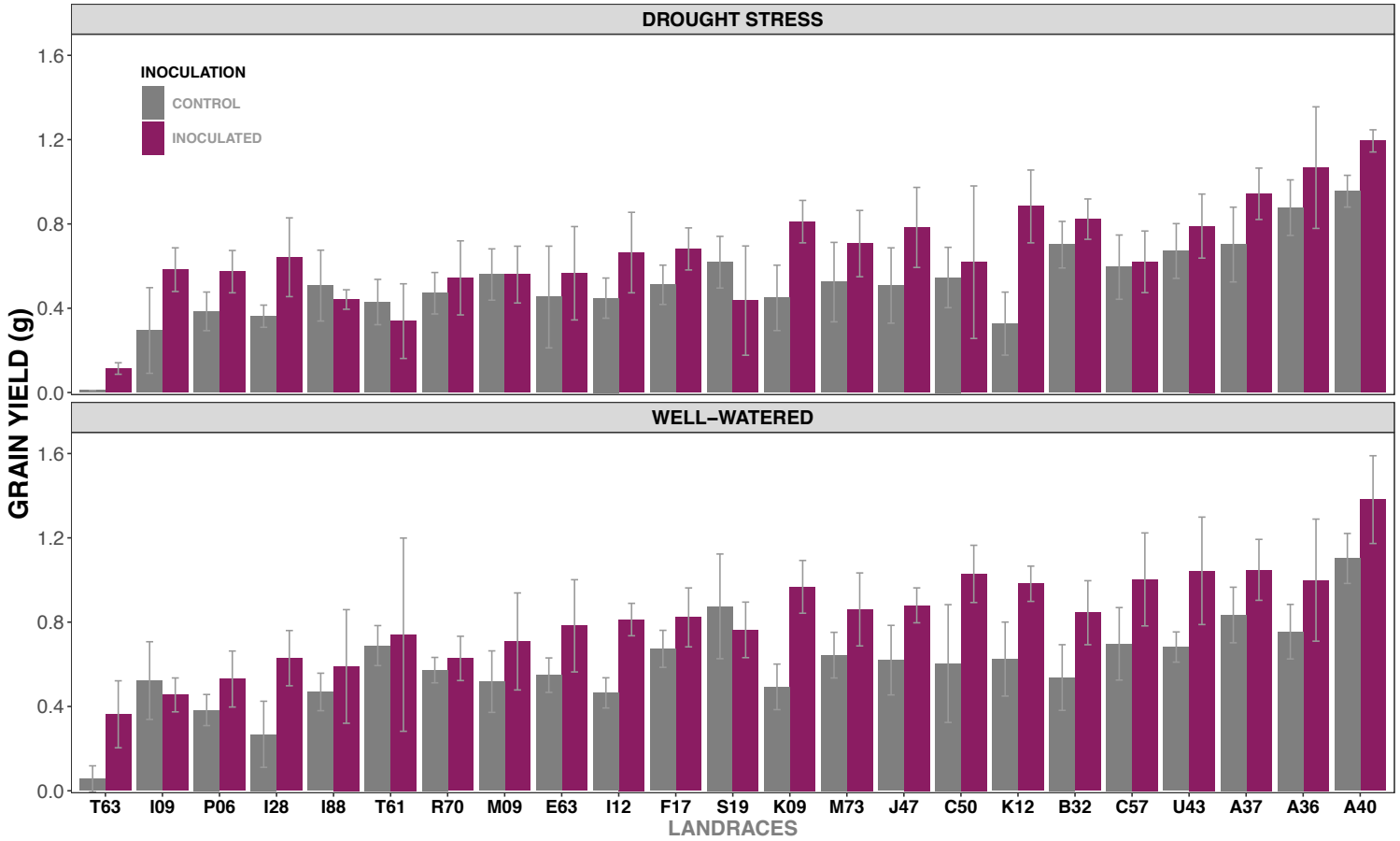


Figure 4.9 | Grain production (g) in 23 spring wheat landraces (means ± SD, *n*= 5) subjected to drought stress or under well-watered conditions at two inoculation levels: inoculated (AMF-inoculated) or control (non-inoculated). Significant differences among treatments were carried out by a three-way ANOVA followed by the Tukey's HSD test ($p< 0.05$).

After determining grain yield, additional plant traits such as spike length per plant, grain number per spike, total grain number per plant, and number of spikes per plant observe its correlation with grain yield (Figure 4.10). This analysis indicated that in most of the wheat landraces, number of grains per spike and the total number of grains per plant are highly correlated with the total grain yield. Moreover, the length of the main spike was positively correlated with the number of grains per spike and, consequently, with overall grain production. The main spike produced a higher number of grains compared to secondary spikes, thus the total number of grains per plant showed a strong correlation with grain yield.

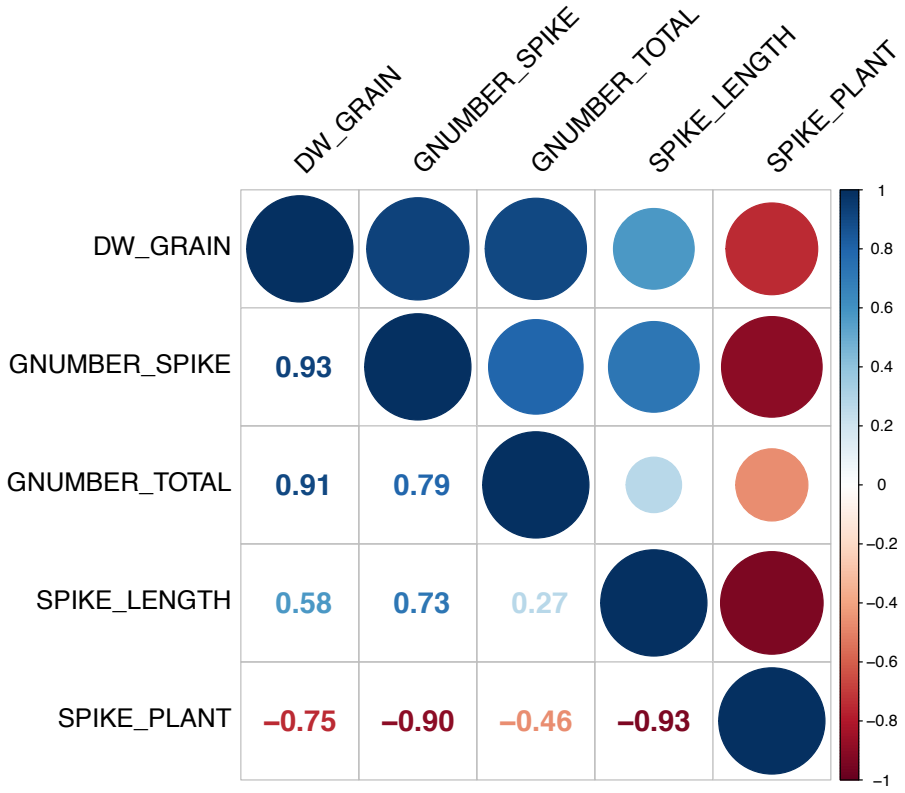


Figure 4.10 | Graphical correlation matrix for grain yield (DW_GRAIN) compared with multiple variables: spike length per plant (SPIKE_LENGTH), grain number per spike (GNUMBER_SPIKE), total grain number per plant (GNUMBER_TOTAL) and number of spikes per plant (SPIKE_PLANT). Larger, darker circles represent stronger correlations, blue indicates a positive correlation, red represents negative correlation. Blue circles indicate a positive correlation and red ones denote a negative correlation.

4.4 Discussion

4.4.1. Physiological and morphological parameters

Wheat plants with spring growth habits have traditionally been classified as unresponsive to vernalisation. However, this classification is not entirely accurate, as some genotypes can exhibit a facultative response to vernalisation, requiring minimal environmental stimulus for flowering (Hyles et al., 2020; Bloomfield et al., 2023). Therefore, the lack of transition to the flowering stage observed in seven of the wheat landraces in this experimental research may be attributed to their need for a certain level of vernalisation. This is suggested by the consistent response of all plants across different inoculation and irrigation treatments, indicating that the vernalisation requirement is intrinsic to these genotypes rather than influenced by external conditions (Jedel et al., 1986; Van Herwaarden et al., 2021).

The vernalisation process in wheat is influenced by several factors beyond just low temperatures, including photoperiod and developmental genes that affect plant growth and development (Foulkes et al., 2020; Wang et al., 2022). It has been demonstrated that mechanical cold treatments can induce vernalisation in some plant species, although the effectiveness of such treatments can vary depending on the genotype (Milec et al., 2023). However, implementing cold treatments for wheat can be labour-intensive and challenging in terms of establishing optimal conditions. Consequently, to simplify the experimental process, we opted to exclude those genotypes requiring cold treatment, narrowing the focus to 23 landraces for further analysis.

4.4.2. Enhanced chlorophyll levels under drought stress

Total chlorophyll measurement using portable, low-cost, non-destructive sampling devices is a common method to evaluate photosynthetic efficiency and environmental stress in crop plants, including wheat. Chlorophyll levels are often used as indicators of plant health, with reduced chlorophyll content frequently

observed in plants under drought stress compared to well-irrigated ones (Zhu et al., 2012).

Overall, we observed that under drought stress, most of the inoculated plant genotypes increased their total chlorophyll levels when compared with non-inoculated controls. These findings are consistently supported by other similar studies (Talaat & Shawky, 2014; de Andrade et al., 2015; Prakash Sharma & Sharma, 2017; Bidellaoui et al., 2019; Sheteiwy et al., 2022), when different crop plants, including wheat, were inoculated with *R. irregularis* strains, finding that the chlorophyll levels were statistically higher compared to non-inoculated treatments under diverse biotic or abiotic stresses.

In this experimental study it was found that under drought stress, most of the inoculated wheat landraces exhibited increased total chlorophyll levels compared to non-inoculated controls. This observation is in agreement with recent studies indicating that inoculation with *R. irregularis* strains often results in higher chlorophyll levels under various biotic and abiotic stresses (Talaat & Shawky, 2020; de Andrade et al., 2021; Prakash Sharma & Sharma, 2021; Bidellaoui et al., 2023; Sheteiwy et al., 2024).

The observed increase in chlorophyll levels under drought stress in inoculated genotypes suggests that these plants may have been more efficient in nutrient and/or water uptake. Since chlorophyll molecules contain significant amounts of nitrogen, the higher chlorophyll levels in inoculated plants could be linked to enhanced nitrogen assimilation. This, in turn, is likely correlated with improved photosynthetic efficiency, as plants with higher chlorophyll content typically exhibit greater photosynthetic rates. Thus, the improved chlorophyll levels in inoculated wheat landraces could reflect a more effective physiological response to drought stress (Smith et al., 2022; Brown et al., 2023).

It has been reported that the photosynthetic rate in AMF-inoculated plants is higher compared to non-inoculated plants, as a result of compensating for the carbon (hexose sugars) transported to them (Gavito et al., 2020). Therefore, it is plausible

that the increased chlorophyll content in wheat plants was not solely a response to *R. irregularis* inoculation, but also a reaction to drought stress, given that well-watered plants exhibited different responses.

The findings in this study aligned with those of Mathur et al. (2021) and Rani et al. (2023), who demonstrated that AMF may employ various mechanisms to mitigate drought effects in inoculated wheat plants. These mechanisms likely involve enhancing water uptake through extraradical hyphae, thereby improving plant water status and, consequently, maintaining chlorophyll levels despite drought conditions.

4.4.3. Above- and below-ground dry biomass under drought stress

Under well-irrigated conditions, 91% of the wheat landraces exhibited increased aerial dry biomass. These results contrast with those reported by Elliott et al. (2021), who found no significant difference in above-ground biomass among three modern wheat varieties, despite using the same commercial inoculum with the AMF species *R. irregularis*. In contrast, the findings in this research are consistent with those of a meta-analysis by Pellegrino et al. (2022), which reviewed the impact of AMF inoculation on wheat. Pellegrino et al. (2022) reported a 12% increase in above-ground biomass in greenhouse experiments with AMF, including *R. irregularis*, compared to controls.

On the other hand, below-ground dry biomass was not uniform across all wheat landraces. Under optimal irrigation, only 30% of the inoculated plants showed a significant increase in root dry matter. Similarly, Elliot et al. (2023) found that inoculation with *R. irregularis* under well-watered conditions led to a significant reduction in root dry matter in two out of three wheat cultivars. This variability highlights the complex interactions between AMF inoculation and root biomass production, suggesting that genotype-specific responses may play a critical role.

The discrepancies between our dry biomass results and those reported by Elliott et al. (2021) may be attributed to differences in experimental design. Elliott et al.

(2021) utilised agricultural soil mixed with sand as the pot substrate, which could introduce a range of beneficial or pathogenic microorganisms not controlled in their study. This variability might obscure the effects of commercial AMF species alone. Additionally, wheat plants in their study were harvested before the completion of their life cycle, potentially limiting the accumulation and translocation of resources from the substrate to the plant, and thus affecting the final dry biomass.

Drought stress was a significant factor negatively impacting dry biomass across most wheat landraces. Inoculation with *R. irregularis* demonstrated differential effects. The above-ground dry matter of 95% of the AMF-inoculated wheat plants was statistically higher, possibly due to various protective mechanisms conferred by the AMF (Yao et al., 2022). Conversely, below-ground dry matter was significantly increased in only 35% of the AMF-inoculated wheat plants. Twenty-two percent of these plants exhibited no significant difference, while the remainder showed statistically lower biomass compared to non-inoculated controls (Smith et al., 2023).

In a meta-analysis, Chandrasekaran (2022) investigated the impact of mycorrhization on various plants, including those inoculated with common AMF species such as *R. irregularis*. Their findings demonstrated that AMF inoculation increased root dry weight by 49% under moderate drought stress (60% FC), a result that contrasts with the observations in this research project. The analysis also highlighted significant positive effects of AMF on total biomass and mineral nutrient uptake (Chandrasekaran, 2022).

4.4.4. Plant height under drought stress

Height in wheat plants is an important trait in breeding programmes. Modern wheat cultivars are typically shorter than landraces to reduce lodging and subsequent grain loss (Denčić et al., 2000). Drought stress has been shown to negatively impact plant height, consequently reducing biomass accumulation. In contrast to well-watered conditions where height (and grain production) usually increase (Gao et al.,

2020). Therefore, it has been suggested a strong correlation between plant stature and total dry plant biomass (Gao et al., 2020; Tena et al., 2021).

4.4.5. Quantification of AMF root colonisation

In general, root colonisation by *R. irregularis* was higher than 50% in the 12 spring wheat landraces. There was no statistical evidence to suggest that drought stress influenced the total percentage of root colonisation. However, analysis of AMF structures revealed that drought stress significantly reduced arbuscule abundance compared with the well-irrigated (inoculated) control.

In a similar experimental approach, Pons & Müller (2022) observed AMF colonisation lesser than 20% in well-watered conditions for two spring wheat cultivars inoculated with *R. irregularis*. Under drought stress, colonisation significantly decreased to less than 10% in both cultivars. Similarly, Pellegrino et al. (2020) reported a percentage of inoculation about 15% with *R. irregularis* in twelve wheat cultivars under well-watered conditions compared to non-inoculated controls. These findings supported the idea of wheat genotype variability in AMF colonisation and in response towards drought stress.

The findings in this experimental research exhibited similarities with those of other studies on well-watered treatments. For instance, Pérez-De-Luque et al. (2017) investigated two wheat cultivars inoculated with *R. irregularis* and reported that the total percentage of inoculation exceeded 75%, while arbuscular abundance was below 20%. Similarly, Elliott et al. (2021) found a total percentage of inoculation (approximately 50 to 80%) in three wheat cultivars inoculated with *R. irregularis*, with an arbuscular abundance ranging from 5 to 30%. These results are consistent with the ones observed here under well-watered conditions, where root colonisation by the same AMF species ranged from 60 to 90%.

The arbuscular abundance percentage across 12 spring wheat landraces, aligned with the findings of other research conducted under well-irrigated conditions, where arbuscular abundance varied between 5 and 35% (Jiang et al., 2021; Smith et

al., 2022). Recent studies support this observation, indicating that drought stress can alter AMF colonisation dynamics and affect the distribution and abundance of arbuscules within the root system. Kumar et al. (2023) found that drought conditions significantly reduced arbuscule density and overall AMF colonisation in wheat, highlighting the detrimental effects of water scarcity on AMF symbiosis. Similarly, Zhang et al. (2023) reported that drought stress diminished AMF colonisation efficiency and affected the spatial distribution of arbuscules in maize, underscoring the broader implications for crop productivity under water-limited conditions.

4.4.6. Quantification of AMF root colonisation

Despite observing a slight positive trend when comparing results from microscopic versus the relative quantification in qPCR of AMF, the results must be interpreted with caution, as a strong positive relationship was not detected using correlation analysis. This lack of a significant correlation suggests that, in our study, the association between the methods may not be linear, as reported by Bodenhausen et al. (2021). Such findings align with recent research indicating that the interaction between AMF and plant dynamics can be complex and non-linear (Smith & Read, 2021; Zhang et al., 2022).

It is plausible that factors related to AMF-plant dynamics are influencing these results. For instance, the variability in AMF colonisation efficiency and its effects on plant growth may contribute to the observed discrepancies (Gosling et al., 2020). Therefore, further analyses involving a larger sample size and more detailed exploration of the AMF-plant interactions are needed to validate these findings and better understand the underlying mechanisms (Rillig et al., 2022).

4.4.7. Grain production

Total plant grain yield is linked to grain size and weight, which are influenced by both intrinsic plant factors and biotic and abiotic interactions throughout the crop life cycle. Grain size is determined by a range of genes that regulate the number of

cells contributing to the final grain size (Morris et al., 2020). Grain weight, similarly, is affected by cell number and processes such as cell division and expansion (Huang et al., 2021).

Moreover, the rate of grain filling, which encompasses starch accumulation and reserve mobilisation, is crucial in determining final grain yield. Abiotic stressors, such as drought, can disrupt these processes, leading to reduced crop productivity (Brinton & Uauy, 2019; Wang et al., 2022). The impact of environmental conditions on grain filling and weight highlights the importance of understanding genetic and physiological mechanisms that underpin grain yield, particularly under changing climate conditions (Zhang et al., 2023).

Inoculation with *R. irregularis* significantly enhanced grain production in most plant genotypes, regardless of the irrigation regime. This effect likely contributed to the overall grain yield. This observation aligns with the meta-analysis conducted by (Pellegrino et al., 2015), where they reported an increase of 18% over the grain yield in AMF-inoculated wheat plants in greenhouse experiments. Similarly, a study by Zhang et al. (2022) on *S. bicolor* demonstrated increased grain production and improved plant nutrition when inoculated with *R. irregularis* under well-watered conditions.

Conversely, Oliveira et al. (2017) found that *R. irregularis*-inoculated *Vigna unguiculata* did not exhibit improved drought tolerance and, in some cases, showed reduced grain production compared to non-inoculated controls. This suggested that the effectiveness of AMF inoculation can vary depending on plant species and environmental conditions.

In our study, the use of commercial inoculum *R. irregularis* provided a notable advantage to the different wheat genotypes under challenging abiotic conditions. This benefit was likely due to enhanced water and nutrient uptake, which positively influenced various morphological traits, including grain yield. However, it is important to consider that different wheat landraces exhibited varying degrees of

drought resistance based on their unique developmental strategies, which could influence their response to AMF inoculation.

4.5 Appendix S4

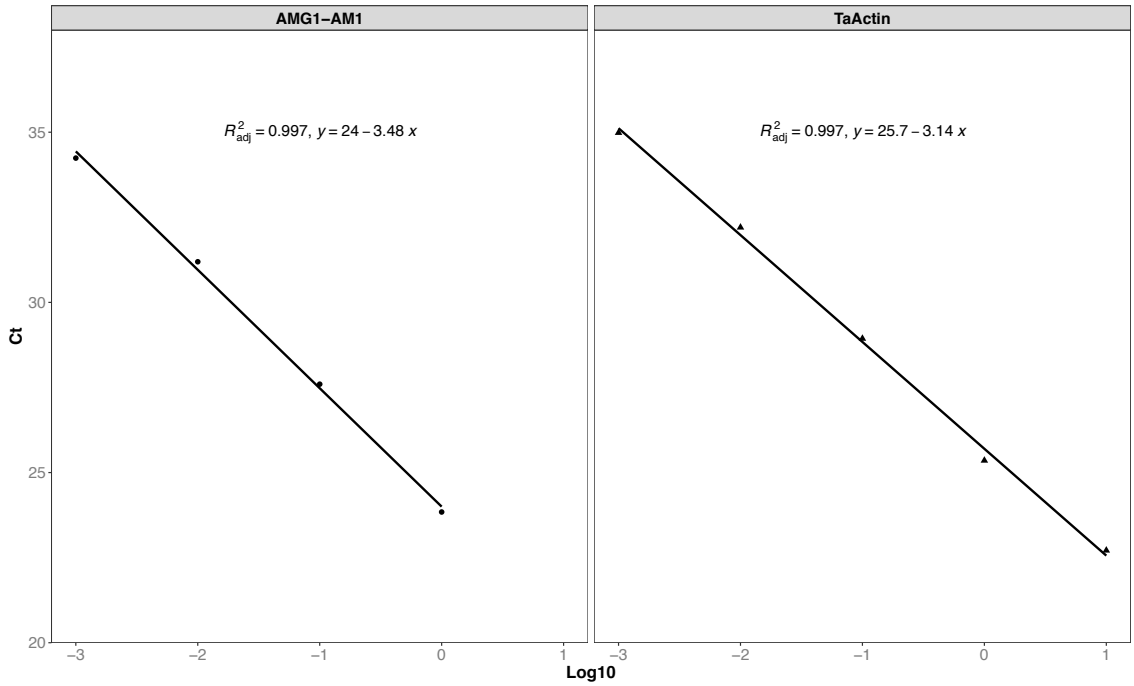


Figure S4.1 | Linear regression of the cycle threshold (Ct) values and the log of the wheat DNA concentrations obtained by qPCR using either the AMG1F-AM1 or TaActin set of primers.

Chapter 5. General discussion

The first research project was focused on measuring various physiological and morphological parameters in four different wheat landraces to evaluate the potential benefits of AMF symbiosis under drought stress conditions in greenhouse settings. The adaptation of local wheat landraces to adverse conditions, particularly in the face of recent climate changes, highlights their value. Their unique morphological and agronomic traits make them suitable candidates for hybridisation with commercial wheat varieties.

Morphological traits can serve as relatively rapid indicators for screening wheat genotypes for grain yield. These traits can be effectively employed in breeding programs to develop new wheat genotypes that exhibit improved performance and adaptability to harsh environmental conditions such as drought stress (Gharib et al., 2021).

We hypothesised that different wheat landraces, inoculated with soil containing AMF propagules, would enhance the ability of the plant to tolerate drought stress and potentially produce drought-resistant genotypes with higher grain yields. Several studies have supported the hypothesis that AMF inoculation can improve drought tolerance and overall plant performance. For instance, research by Ahanger et al. (2021) demonstrated that AMF colonisation can enhance water use efficiency and reduce oxidative stress in wheat under drought conditions. Similarly, the research by Liu et al. (2022) emphasised that mycorrhizal associations can mitigate the adverse effects of drought on wheat plants, aligning with the results observed in a wheat cultivar.

As part of the same research project, we aimed to evaluate various morphological and physiological traits in four wheat landraces to assess how AMF and drought stress might influence these parameters. Given that AMF are known to enhance plant protection, it was crucial to investigate how these fungi could mitigate the adverse effects of drought stress on wheat.

It has been demonstrated that AMF colonisation increases plant resilience under water-limited conditions, by using various mechanisms, which is crucial for maintaining yield stability (Khan et al., 2021). For instance, research by Zhang et al. (2022) highlights that AMF inoculation can alleviate drought-induced stress by improving nutrient uptake and reducing oxidative damage.

The results presented in Chapter 2 offered valuable insights into the complex interactions between wheat landraces, AMF inoculation, and drought stress. The findings indicated a generally positive outlook regarding the potential benefits of AMF inoculation for wheat landraces under drought conditions. This preliminary analysis highlighted the potential role of AMF in enhancing drought resilience in wheat, suggesting that certain landraces may benefit more from AMF inoculation than others.

Given the relatively low level of AMF colonisation observed at the time of root sample collection, it was decided to use the genetic material from these samples to further investigate the fungal community. To gain deeper insights into both AMF and other associated fungi, including potential mutualists or pathogens, we employed next-generation sequencing technologies.

By isolating and sequencing the fungal genetic material, we aim to identify and characterise the different fungal species present in the wheat roots. This included both AMF and other fungal taxa that may play roles in plant health and stress responses. The sequencing data was meant to facilitate the profiling of the fungal community, to assess changes in community structure in response to AMF inoculation and drought stress.

One of the challenges encountered during this experimental project was the potentially low quality of DNA obtained from the wheat root samples, particularly under drought stress conditions. Despite using next-generation sequencing technologies, it was not possible to obtain a representative and accurate classification of AMF and other fungal species across the different treatments.

Due to these limitations, the fungal community composition was not fully characterised, however, they provide a useful representation of the fungal communities in the spring wheat landraces under glasshouse conditions and importantly, in response to drought stress. This challenge underscored the complexity of working with AMF and fungal communities in stressed environments and emphasises the need for robust methodologies to ensure the successful isolation and analysis of microbial genetic material.

Moving forward, efforts focused on adopting a different approach, which involved including a broader variety of wheat landraces and AMF strains. This was intended to provide a better understanding of their interactions and to optimise inoculation strategies. By refining the experimental design to address these aspects, we aim to gain clearer insights into the potential benefits of AMF inoculation for wheat cultivation in potentially drought-prone areas, ultimately contributing to more resilient agricultural practices.

The results from Chapter 4 demonstrated successful AMF colonisation in all inoculated wheat landraces with the commercial AMF inoculum, which had a general positive influence on the above-ground dry biomass of wheat landraces under drought stress. However, the below-ground biomass did not exhibit a consistent response to either inoculation or water stress. Interestingly, the physiological parameter of chlorophyll content showed a positive correlation with drought stress, regardless of the inoculation status.

Moreover, root colonisation was evaluated in 12 wheat genotypes using a novel approach: relative quantification via qPCR, compared with the standard microscopic method. The sample-to-sample comparison between the methods revealed a positive correlation, although it was not linear, this contrasted with findings by Bodenhausen et al. (2021), who reported a linear positive correlation between the two methods.

Remarkably, inoculation with *R. irregularis* significantly enhanced grain yield in nearly all wheat genotypes, even under drought stress. These findings align with

those in a meta-analysis by Pellegrino et al. (2015), which reported similar patterns of improved grain yield in AMF-inoculated wheat plants under greenhouse conditions. The use of AMF, either independently or in combination with other beneficial microbes, represents a promising opportunity in agricultural research. The evidence from previous studies emphasises the significant benefits of AMF in enhancing crop nutrition, growth, and yield, as well as improving soil quality and biological fertility. Moreover, AMF symbiosis plays a crucial role in increasing plant resistance to pathogens and boosting tolerance to abiotic stresses, specifically drought stress. These findings highlight the potential of AMF to contribute to more sustainable and resilient agricultural practices (Kavamura et al., 2021).

5.1 Conclusions

An important trait in breeding programmes is grain yield and its components. In this study, virtually all wheat landraces under well-watered conditions exhibited significantly higher grain production compared to non-inoculated treatments. Furthermore, under drought stress, AMF-inoculated plants outperformed their non-inoculated counterparts, with this improvement strongly correlated with the number of grains and potentially their size. These findings suggest that drought stress negatively influenced both grain number and, in some cases, grain size across most wheat landraces.

Therefore, the application of commercial inocula (or single AMF species) to diverse crop plants across different agricultural systems, including field and greenhouse environments, could offer substantial benefits. Specifically, in spring wheat landraces, AMF inoculation has the potential to provide protection against moderate drought stress (60% field capacity), ultimately enhancing their production.

5.2 Future work

Current work has focused on investigating how arbuscular mycorrhizal fungi influence wheat landraces in coping with drought stress, by assessing various physiological and morphological plant parameters, as well as certain morphological characteristics of the fungi. Two inoculation sources were used across two experimental studies, each presenting distinct advantages and disadvantages, yet both approaches were well-suited for achieving the study's objectives. However, several questions remain unanswered based on our findings, warranting further investigation.

1. From the first experimental study, we observed that virtually all spring wheat landraces exhibited increased chlorophyll levels under drought stress, contrary to the lower levels commonly reported in the literature. It would be valuable to formulate hypotheses to explore the mechanisms underlying this

response and to evaluate the influence of both biotic and abiotic factors on this crucial physiological trait.

2. In the second experimental study, we standardised the primers AMGF1-AM1 and TaActin for the relative quantification of AMF root colonisation in wheat through qPCR. This methodology could be extended to various wheat varieties, including both modern and historical strains, as well as related species, to determine if a positive correlation is observed. This approach may offer advantages over the traditional microscopy methods.

3. The second experimental study demonstrated that, under optimal irrigation, wheat landraces showed increased production compared to non-inoculated controls. Similarly, inoculated plants performed better under drought stress than their non-inoculated counterparts. It would be valuable to replicate this system in field conditions with two different irrigation regimes to determine if the spring wheat landraces exhibit similar behaviours or if the microbiome alters the dynamics between plants and AMF.

Finally, our findings represent an initial approach to evaluating the performance of wheat landraces under drought stress. They provide a basis for assessing the potential improvements in grain production when these landraces are inoculated with one or more AMF species. Such insights could be valuable for enhancing plant breeding programmes.

Chapter 6. References

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