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# Genetic characterization of tetraploid wheats and evaluation of their mycorrhizal affinity

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**Summary**

Genetic diversity of domesticated wheats has been significantly reduced compared to that of their wild progenitors, through a long selection procedure for those phenotypic traits which led the wild plants to better suit the human needs. Tetraploid wheat landraces were largely cultivated until the first decades of the twentieth century, being progressively abandoned from the early 1970s and replaced with improved, genetically uniform semidwarf cultivars as a consequence of the Green Revolution. Nevertheless, since the current climate change is affecting grain yields worldwide and threatening food securety, sources for specific adaption to drough and heat are urgently needed. In this context, addressing the research towards the study of the level and the structure of genetic diversity in tetraploid wheats, linked to the dection of specific chromosomic traits of interest, has become very important. The relatively high level of genetic variation in modern crops could be obtained through the genetic drift and introgressions between or among the domesticated crops and their close wild relatives. In particular, landraces, characterized by a wide variability in terms of morphological, phenological and quality traits, provide a large source of genetic variability. Many researches have showed their specific adaptation to local environmental conditions according to their place of origin, and, very recently, their ability to form mycorrhizal symbiosis. Positive advances have been reported regarding the mutualistic relationship between the plant and the mycorrhizal fungus, revealing better performance for the host in terms of nutrient uptake and protection from salinity, lack of water, and excess phytotoxic elements. Mycorrhiza studies and the recent progress in research in this sector have shown a possible solution for environmental sustainability: AMF represent a valid alternative to overcome the loss of biological fertility of soils, reduce chemical inputs, and alleviate the effects of biotic and abiotic stresses. However, the actual role of the single wheat genotype in establishing this type of association is still poorly investigated. In this work, the genetic diversity and population genetic structure of a collection of 265 accessions of eight tetraploid *Triticum turgidum* L. subspecies were investigated using 35,143 single nucleotide polymorphisms (SNPs) screened with a 35K Axiom® array. Neighbor joining algorithm, discriminant analysis of principal components (DAPC) and Bayesian model-based clustering algorithm implemented in STRUCTURE software revealed clusters in accordance to the taxonomic classification, reflecting the evolutionary history and the phylogenetic relationships among *Triticum turgidum* L. subspecies. Starting from these results, 130 accessions have been inoculated with the AMF species *Funneliformis mosseae* (*F. mosseae*) and *Rhizoglomus irregulare* (*R. irregulare*), and a genome wide association study (GWAS) was conducted to identify genetic markers in linkage with chromosome regions involved in this symbiosis.

Six clusters of genetically related accessions were identified, showing a different mycorrhizal colonization among them. GWAS revealed four significant quantitative trait nucleotides (QTNs) involved in mycorrhizal symbiosis, located on chromosome 1A, 2A, 2B and 6A. The results of this work enrich future breeding activities aimed at developing new grains on the basis of genetic diversity on low or high susceptibility to mycorrhization, and, possibly, maximizing the symbiotic effects.

# CHAPTER 1

# General introduction

## 1.1 Tetraploid wheats

### 1.1.1 Wheat agronomic importance and botanical classification

Wheat (*Triticum* ssp.) represents a stable food for millions of people, being one of the most cultivated crop in the world with a global production of 732.5 million tons in 2018/2019 [1].

It provides nearly 55% of carbohydrates and 20% of the food calories [2], and it is widely used for bakery and pasta production, thanks to the extensibility and elasticity lend by its gluten-forming proteins [3]. A total of 55% of processed wheat flour - made from the grinding of this cereal - is estimated to be used in the baking and confectionery industry, while the 17%, 15%, 11% and 2% are respectively used for domestic consumption, dough, cookies and animal feeding [4].

According to the van Slageren (1994) system [5], currently judged to be the most up-to-date taxonomic classification by the European Wheat Consensus Document (OECD, 1999), wheat is arranged within the *Angiospermatophyta* phylum, the *Monocotyledonopsida* class, the *Poales* (*Glumiflorae*) order, the *Poaceae* (*Gramineae*) family, the *Pooideae* subfamily , the *Triticeae* tribe, the *Triticinae* subtribe and the *Triticum* L. genus [6].

Seven is the chromosome number of all species of *Triticum* genus which is divided in three ploidy levels:

i) diploid series: 2n = 2x = 14, genome $A^m$ or A (*T. monococcum*, *T. urartu*);

ii) tetraploid series: 2n = 4x = 28, AB (*T. turgidum*) or AG (*T. timopheevii*);

iii) hexaploid series: 2n = 6x = 42, ABD (*T. aestivum*) or $AGA^m$ (*T. zhukovskyi*).

### 1.1.2 Origin and domestication of *Triticum* L. species

Wheat cultivation occurred around 10,000 years ago when human beings started to shift from hunting and gathering to self-production [7]. Wheat species origin is still today much discussed by scientists, even if the beginning of their evolution have been corresponded by many studies with the separation of a diploid prototype in the *Triticum* and *Aegilops* genera ancestors (Fig. 1) [8, 9]. From the *Triticum* genus, two wheats species seemed to have evolved in parallel, *T. urartu* (A), which have been recognized as the A genome donor [10], and *T. monococcum* (einkorn) ($A^m$). This last diploid species was the first

wheat to be cultivated successfully and it has been domesticated from its wild progenitor *T. boeticum* [11], whose kernels have been found in Epi-Palaeolithic and early Neolithic sites of the central Fertile Crescent [12]. Karacadag mountains of southeast Turkey [13], Jordan basin [14] and southeastern Turkey [15] have been indicated as domestication sites for *T. monococcum*, highlighting a results divergence among the conducted researches. Anyway, the cultivation of einkorn was abandoned during the Bronze Age, and replaced by polyploid wheats. The origin of B genome is uncertain but most of researches have referred to tetraploid wheats creation (AB) the hybridization between a species related to the *Sitopsis* section of the *Aegilops* genus (S) and *T. urartu* [16]. Among the *turgidum* species, *T. turgidum* ssp. *dicoccoides* (wild emmer), characterized by brittle ears that shatter at maturity into spikelets which bear relatively large seeds, have been defined the ancestor of the other cultivated species [17]. Today, among the free-threshing wheats derived from their wild progenitor *T. turgidum dicoccoides*, *T. turgidum durum* is the only one that is widely cultivated, nevertheless, the domestication of *T. monococcum* (emmer) has signed an important step in the evolution of polyploid wheat varieties. This species, with hulled seeds and the AABB genom, was the most important crop in the Fertile Crescent until the early Bronze Age [18].

Finally, the hybridization of *Ae. tauschii* (D) - the D genome donor – with *T. turgidum* ssp. *dicoccon* - originated the hexaploid wheat [19], whose genome (ABD) represents one of the largest crop genomes with approximately 15 billion pare pairs. Among hexaploid wheats, *T. aestivum* ssp. *spelta* has been considered the most ancient subspecies [20], from which the naked types (*T. aestivum* ssp. *aestivum*, ssp. *sphaerococcum*, ssp. *compactum*) were developed later [21]. *Ae. speltoides* (S) and *T. urartu* may have been the ancestors of the tetraploid *T. timopheevii* (AG), whose hybridization with *T. monococcum* (Am) may resulted in the species T. zhukovskyi (AGAm) [22].

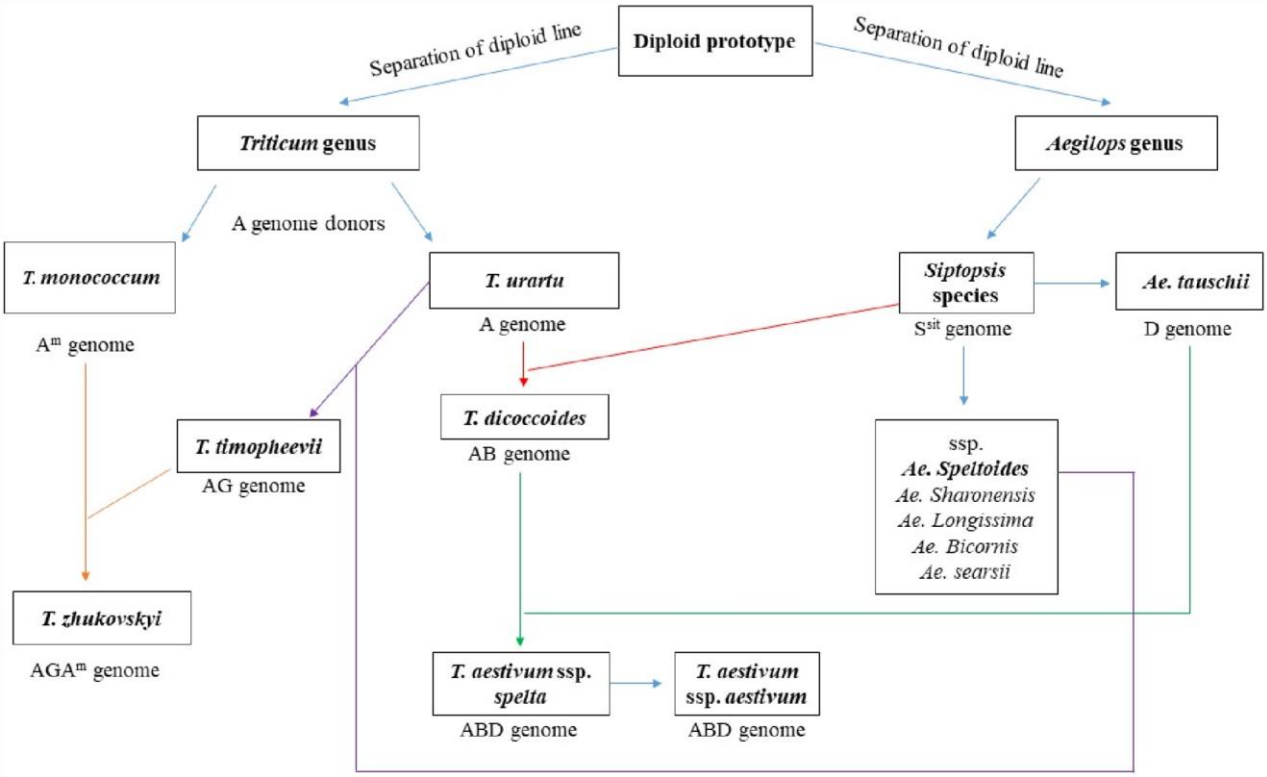


**Fig. 1. Summary scheme of *Triticum* L. species origin**

### 1.1.3 History of *Triticum turgidum* ssp. *durum* breeding in the Mediterranean Region

Wheat breeding started with the first selections from *T. turgidum* ssp. *dicoccoides* wild emmer populations, basing primary both on yield and morphological characteristics, such as non-brittle rachis and free-threshing naked kernels [7]. In the seventh millennium BC, the cultivated emmer - *Triticum turgidum* ssp. *dicoccum* -represented the first dominant wheat in Asia, Africa, and Europe [23] and it remained a dominant cereal until the first millennium BC when it was replaced by free-threshing durum wheat. Wheat breeding programs started in the early 20th century within public research institutes of Mediterranean basin, basing initially on landraces material and successively on high yield and diseases-resistant cultivars. Regarding Italy,

wheat breeding has begun in public research institutes, universities, and departments from the Ministry of Agriculture, and continued later within private companies, such as Società Italiana Sementi and Società Produttori Sementi Bologna [24]. Through the selections and the interspecific hybridizations of local landraces from southern Italy, the Italian islands, and the Mediterranean Region, many efforts for durum wheat breeding have been done by some famous Italian breeders, such as Nazareno Strampelli (1866–1942), Francesco Todaro (1864-1950) and Roberto Forlani (1902-1953), leading to new cultivars constitution [25, 26]. Concerning Greece, wheat breeding started in 1923 in the Institute of Plant Breeding, founded by Ioannis Papadakis (1903-1997), which conducted local breads and durum wheat landraces selections and crossbreeding [27]. In France, wheat research was initially performed by the Institut National de la Recherche Agronomique (INRA) in Montpellier (until the 1962) and then by The Algerian Center for Agronomic, Scientific, and Economic Research (CARASE), or INRA, in Algeria [28]. The selections among local landraces have led to new cultivars release which considerably increased durum wheat production. French breeding work has been also conducted in Morocco and Tunisia, where collection and selection of landraces resulted in durum cultivars constitution [29, 30]. Spanish selection and crossbreeding programs have been performed at the Agricultural Research Center of Jerez de la Frontera by Juan Bautista Camacho [31], while wheat breeding in the North Africa took place in Egypt (1914) and Libya (1950s) which resulted in improved and high temperature-resistant varieties [32,33]. Regarding the eastern part of Mediterranean Region, in the 1920s, the Seed Improvement Station (located in Turkey) and the Israeli wheat improvement programs released durum wheat cultivars from local durum wheat populations selection [34].

In the 1960s, the “Green Revolution” involved the introduction of semi-dwarf genes in wheat, which mostly affected the plant height and the elimination of photoperiod response. Tall cultivars were replaced with semi-dwarf cultivars, characterized by short stature and high responsiveness to fertilizers. Two important research centers, Centro Internacional de Mejoramiento de Maiz y Trigo (CIMMYT) in Messico and the International Center for Agricultural Research in the Dry Areas (ICARDA) in Syria have played a key role in developing countries breeders activities, including the revealing of new crop varieties. Accompanying these changes in plant morphology, productivity has

been increased in grains per square meter. In Italy, comparing old (<1945), intermediate (1950–1985), and modern (1988–2000) cultivars, the number of grains per $m^2$ in modern cultivars has increased by 39%.

## 1.2 Genetic diversity of wheat accessions

### 1.2.1 Use of landraces for wheat breeding programs

Genetic diversity of wheat germplasm has suffered an overall reduction over time as a consequence of their replacement by high-yielding varieties. Tetraploid wheat landraces have been largely replaced by monocultures of pure genotypes and this genetic erosion has resulted in a significant loss of valuable genetic diversity of quality traits and resistance or tolerance to biotic and abiotic stresses.

Recently, genetic diversity and tetraploid wheats evaluation structure received a lot of attention [35-37], especially for new and unpredictable environments [38]. Collecting, replenishing, reproducing, studying and maintain those species living has become important to preserve biodiversity resources and future food security. Landraces represent a good reservoir of genetic variability for wheat breeding programs, being valuable sources of useful agronomical traits for the cultivated species improvement. Involving wild and non-cultivated tetraploid species in interspecific hybridization for introgression of genes or/and their alleles into cultivated species could be a way to solve the problem of increasing durum wheat yields under rainfed conditions.

Before and during molecular markers spreading, genetic diversity and population structure of wheat species have been assessed through different criteria. Genetic variability analysis based on pedigree record have been performed on wheat genotypes [39,40], even if many authors have showed a lower efficiency for pedigree-based diversity measurements when compared to molecular markers [41-43]. Morphologic markers have been widely used in many researches, including the works of Máric et al. (2004) [44] on 14 cultivars, Masood et al. (2005) [45] on 298 wheat landraces, Kotal et al. (2010) [46] on 14 genotypes and Rizwana et al. (2010) [47] on 100 spring wheat genotypes. The morphological traits considered in the analysis were represented by plant spikes number, plant high, harvest index, grains number and weight, etc. Finally, wheat accessions characterization have been

performed with cytological [48] and biochemical markers, such as grain proteins [49] and isozymes [50].

Nevertheless, these markers showed some disadvantages, such as their limited number and the plant growth stages and/or environmental factors influence. During the last three decades, molecular genetics and plant breeding programs efficiency were completely revolutionized thanks to the introduction of molecular markers which became a convenient tool to assess genetic diversity in germplasm collections.

### 1.2.2 Molecular markers for wheat breeding

In recent years, a considerable attention was given to molecular markers concerning two major applications in plant system: the development of genetic and physical chromosome maps, and the traits of interest selection for plants breeding improvement. In addition to this aims, DNA markers have been developed also for germplasm characterization and phylogenetic analysis.
Generally, molecular markers are divided in three main groups:

1) hybridization-based DNA markers, such as restriction fragment length polymorphisms (RFLP) and oligonucleotide fingerprinting;
2) PCR-based markers, such as amplified fragment length polymorphisms (AFLPs), random amplified polymorphic DNAs (RAPDs); inter-simple sequence repeat amplification (ISA), simple sequence repeats (SSRs), amplicon length polymorphisms (ALPs), sequence-tagged sites (STS) and cleaved amplified polymorphism sequences (CAPS);
3) sequencing-based markers, such as single nucleotide polymorphisms (SNPs).

Over more than twenty years, molecular markers were used in wheat genome analysis.

Regarding the first group, RFLPs were the most popular molecular markers in late eighties, being widely used in plant genetics thanks to their reproducibility and codominance. They have been adopted for mapping wheat genotypes, including *Triticum aestivum* [51-53] and *Aegilops tauschii* [54] accessions. Despite the expensive and time-consuming process to detect these markers,

RFLPs have been developed also for the selection of chromosomal regions carrying useful genes in wild wheats relatives [55,56] and for 54 Italian wheat cultivars identification [57]. However, generically, only in few cases RFLPs have been used for segregation analysis in wheat breeding, mainly due to the fact that a low level of polymorphism identified by them. The abundance and the uniformly distribution of oligonucleotide fingerprinting, belonging to the same hybridization-based DNA markers group, made it possible the genetic characterization of plant varieties and the introgression of individual genes by backcross breeding [58]. Nevertheless, when used for bread wheat varieties, also these markers revealed a low polymorphism level [59].

Concerning PCR-based markers, RAPD technology has proved useful for gene tagging in durum wheat [60] and for mapping the einkorn wheat [61]. Moreover, these markers have been used for polymorphisms analysis among wild and cultivated tetraploid wheats [62], for genetic diversity evaluation of fourteen Croation wheat varieties [44], and for wheat traits of interest detection, including yield traits [63] and flag leaf senescence [64]. Similarly to RAPDs, STSs have been used to identify chromosome traits of interest, such as those associated to preharvest sprouting tolerance in wheat [65]. AFLP analysis, which showed an higher efficiency in detecting polymorphism when compared to RFLP and RAPD [66-68], has been conducted to assess genetic diversity in wheat cultivars [39] and to construct wheat molecular maps [60,69]. Nevertheless, AFLP markers did not find widespread application in molecular breeding owing to the lengthy and laborious detection method. Despite the expensive cost of detection, SSR markers were used in all areas of plant molecular genetics and breeding in late 90s and the beginning of 21st century. Regarding wheat, the first large set of microsatellites markers for the wheat genome was published in 1988 [70] using 279 microsatellites loci. The high level of variability detected by these markers has led to a broad use of SSRs in many works, including the polymorphism detection in yellow rust-resistant accessions of *T. dicoccoides* [71], and the genes and quantitative trait loci (QTLs) identification for striperust resistance [72], preharvest sprouting tolerance [65], and grain protein content [73]. Moreover, many works focused on genetic diversity study have been conducted with SSRs markers, such as those of Roussel et al. (2005) [74], on a set of 559 French wheat accessions (including landraces and commercial varieties evolved between 1800 and 2000), Wang et al. (2007) [75], on 52 accessions of Xinjiang, Tibetan and

Yunnan wheat, Ijaz and Khan (2009) [76], on 48 Pakistani wheat accessions, and Zhang et al. (2010) [77], on 205 elite breeding lines of winter wheat.

More recently, a shift towards the use of Single Nucleotide Polymorphisms (SNPs) markers occurred. Although the lower level of polymorphism detected because of their biallelic nature, SNPs abundance, ubiquity and amenability to high- and ultra-high-throughput automation, have gained a considerable interest. SNPs are single base-pair changes in the DNA sequence that occur with high frequency and typically have two alleles, meaning that, within a population, there are two commonly occurring basepair possibilities for a SNP location. The frequency of a SNP is given in terms of the minor allele frequency (MAF) or the frequency of the less common allele. With the recent introduction of Next-Generation Sequencing (NGS) technologies, such as 454 Life Sciences (Roche Applied Science, Indianapolis, IN), HiSeq (Illumina, San Diego, CA), SOLiD and Ion Torrent (Life Technologies Corporation, Carlsbad, CA), SNPs discovery has become rapid and less expensive, avoiding the highly repetitive nature of the plant genomes [78]. SNPs markers have been widely used for wheat to reach three main aims: the identification and localization of loci that affect genetic variation or of regions affecting a loci linked to a quantitative trait, the detection of genotypes with a favorable genetic makeup for the purpose of selection, and the assessment of genetic differentiation of individuals or populations. In the recent years, the relationship between genetic polymorphism within a species and the phenotypic differences between genotypes - known as genetic mapping - held a great appeal. From the agronomic point of view, understanding the genetic basis which underlie a phenotype, such as the yield or the growth rate, can help us to efficiently utilize these diverse genetic resources for crop improvement.

## 1.3 Mycorrhizal symbiosis in wheat

### 1.3.1 Arbuscular mycorrhizal fungi (AMF) - plant interaction

Arbuscular mycorrhizal fungi (AMF) are soil-borne fungi for the most part belonging to *Glomeromycota* phylum and forming mutualistic symbiosis with the roots of the plants. The life cycle of AMF, which obligatory needs the host plant to be concluded, starts with the germination of spores and the hyphae production as consequence of their exposition to some physical factors, including pH, temperature and moisture [79]. During this presymbiotic phase,

in presence of host plant root exudates, hyphal branching starts to develop until coming into contact with the host root and forming appressoria. From this fungal organ hyphae penetrate within the root cortex and cells and give rise to the highly branched hyphal tree-like characteristic structures of AMF, the arbuscules. Arbuscules represent the site for fungus/plant metabolites exchange: the host receives mineral nutrients from the fungus while the fungus acquires carbon and lipids from the plant. Thus, mycorrhizae represent mutualistic symbiosis where both plant and fungus take advantage from this relationship (Fig.2). Many studies have revealed the ability of fungal mycelial network to extend in the soil - in the deepest parts where the roots of the plant are unable to reach – and to promote nutrients uptake [80,81]. Mycorrhizal plants, having an higher concentration of macro and micro-nutrients, have showed an increased photosynthate production and, consequently, an higher biomass accumulation. The efficiency of mycorrhizal fungi in the absorption of nutrients has been also observed in the presence of poor soils: under phosphorous-limited conditions, plants colonized by AMF have showed an higher concentration of this mineral [82,83], as well as, under conditions of low soil N levels, mycorrhizal symbiosis has exhibited an improved ability of plants to compete with saprotrophs for the N uptake [84].

Increased levels of crops quality have been ascribe to AMF, resulting in higher concentration of sugars, organic acids, vitamin in C and flavonoids in citrus fruit [85], and in enhanced accumulation of carotenoids, soluble phenolic compounds and anthocyanins in lettuce [86] and tomato [87]. In addition to quality parameters, also the crop yield has appeared to be increased in many different mycorrhizal plants, such as maize [88], potato [89], soybean and cotton [90].

Copious pants benefits derived from this association have been detected under biotic and abiotic stresses conditions. Drough stress, for example, seemed to be alleviated in plants, principally due to the fungal ability to explore the soil and reach higher and farther water sources [91], but also thanks to fungal regulation of a variety of physio-biochemical processes which represent drought adaptive strategies [92,93]. Moreover, positive effects of AMF in alleviating the deleterious effects caused by salinity stress have been reported. Plants growth and yield, normally affected in presence of this stress, have been improved in mycorrhizal plants [94] and beneficial effects of AMF on physiological parameters, such as stomatal conductance and photosynthetic

rate, have been described [95]. Similarly to drought stress, mycorrhizal plants under salinity stress have exhibited higher biomass production and, in addition, increased accumulation of P, Ca2+, N, Mg2+, and K+ [96]

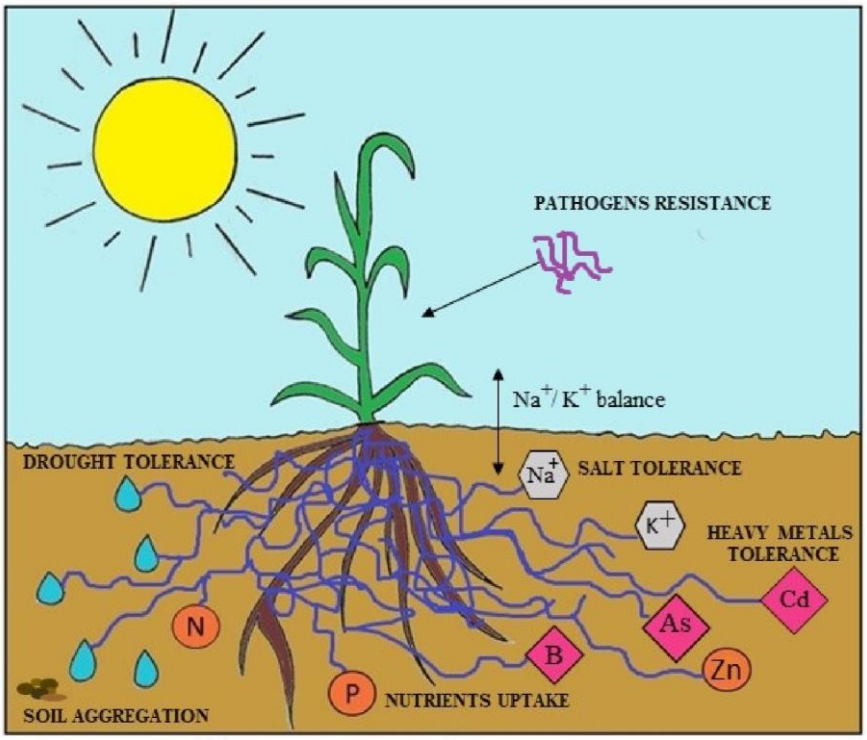


**Fig. 2. Arbuscular mycorrhizal fungus – plant symbiosis**

### 1.3.2 Influencing factors for mycorrhizal colonization

Mycorrhizal dependency has been defined by Gerdemann (1975) [97] as the degree to which a plant is dependent on the mycorrhizal condition in order to produce its maximum growth or yield at a given level of soil fertility. The susceptibility of plants to AMF is highly variable and depends on many factors which have been abundantly debated.

First of all, the influence of environmental factors to explain AMF colonization variability has been assessed. Concerning soil properties, pH was found to have direct correlation with AMF colonization, showing an increased level of symbiosis at a pH level above 7 [98]. Contrary, low AMF colonization rates have been found with high contents of soil organic nitrogen (ON) and high P availability [99]. Regarding climatic variables, higher percentages of AMF symbiosis have been related to lower rainfalls [100], principally due to soil humidity decrease and oxygen concentration increase, which promote spore

germination and growth [101]. In addition, it has been observed that also soil moisture affects mycorrhization. The recent work of Shukla et al. (2013) [102] has registered the maximum symbiosis level in agroforesty pants with field capacity (FC) = 16%.

Secondly, root anatomical traits, including the thickening of the cell walls of the rhizodermis, exodermis and outer cortex or the presence of aerenchyma in the inner cortex, may affect mycorrhizal colonization [103].

Finally, the identity of the inoculum has shown a significant influence on AMF symbiosis [104].

While AMF dependence from environmental, root anatomical traits and inoculum factors have been widely discussed, scanty information regarding best genotypes for mycorrhizal symbiosis are available. Nevertheless, the key role of genotype identity in determining the success of mycorrhizal symbiosis has been recognized for many plants, such as olive [105], and grape [106]. Concerning wheat, genotypic differences have reflected a great variability in mycorrhizal root colonization [107,108]. Wide percentage ranges of mycorrhizal symbiosis have been confirmed - 24-56% in winter wheat, 2.2–21.2% in durum wheat; 1.0–12.0% in domesticated emmer; 0.7–7.1% in wild emmer [109,110] highlighting a significant genotype X AMF interaction. Specifically, different levels of mycorrhization have been observed in genotypes with different geographical origin, year of release, ploidy number and nutrient use efficiency [108, 111]. The possible loss of susceptibility to mycorrhization in modern varieties is today a topic of great debate. Some studies have suggested a lower ability to form symbiosis by high-yielding genotypes, when compared to old varieties [112-114]. Contrary, the research of Ellouze et al. (2016) [115], conducted on 27 cultivars of durum wheat, has revealed how wheat plant breeding in Canada has increased the mycorrhizal development in wheat grown at low soil fertility. More recently, De Vita et al. (2018) [110] rejected this idea, reporting that modern plant breeding programs didn't lead to the suppression of AM colonization.

## 1.4 Quantitative trait loci (QTLs) detection

### 1.4.1 QTLs mapping

Many important agronomic traits in plants, such as yield, disease resistance and flowering time, are controlled by many genes. The genome regions

containing these genes associated with the specific quantitative trait are known as quantitative trait loci (QTLs), while the process to identify these chromosomal regions through the use of molecular markers is called *QTL mapping*. QTL mapping which requires three steps: 1) mapping population screening; 2) linkage maps construction; 3) QTL-trait analysis conduction. For the first step, the method requires a segregating population and the selection of parents which differ in one or more traits of interest. There are several different populations used in mapping (F2, back crosses, recombinant inbreed lines, double haploids) and their choice depends on same factors, such as plant species, the markers and the trait to be mapped. Once the population has been chosen and its size has been defined, – usually from 50 to 250 individuals but maybe larger in high resolution mapping studies-, polymorphic markers have to be identified and screened across the entire mapping population, including parents. Moreover, the selected population has to be phenotypically examined for the trait of interest.

The second step concerns the construction of a linkage map that, according to Peterson et al., (1996) [116], can be considered as a road map of the chromosomes derived from two different parents, where the position and the relative genetic distances between markers along chromosomes are indicated. QTL mapping is based on the principle of genetic linkage, which is the tendency of genes and markers to segregate together through chromosome recombination or crossing over during recombination. Markers and genes which are genetically close together are more likely inherited together from a parent to its progeny. The frequency of recombinant genotypes, which belong to the segregating population, can be used to calculate the recombination fractions, which allow to deduce the genetic distances between markers. Based on this concept, two markers are assumed to be close and located in the same chromosome if the recombination frequency between them is low.

The last phase relates to QTL analysis, focused to detect an association between phenotype. Significant differences in genotypes for a specific measured trait are studied and the relation between these differences and the presence/absence of a particular marker locus is investigated. If there is a significant difference in the phenotypic means of the genotypes with a specific marker, this indicates that the marker is linked to a QTL affecting the trait. Moreover, the QTL and the marker are genetically close or tightly linked and will most likely be inherited together.

Many statistical methods have been developed for QTLs mapping, including Single-Marker Analysis (SMA), Simple-Interval Mapping (SIM), Composite-Interval Mapping (SIM), Composite-Interval Mapping (CIM), MultipleInterval Mapping (MIM) [117-120].

### 1.4.2 Genome-wide association study (GWAS)

In *association mapping* each marker is tested for an association with a particular trait. The assumption behind this method is that significant associations emerge when the marker in linkage disequilibrium with the trait. Association mapping usually refers to two main categories: 1) candidate-gene association mapping, which focuses on associations between genetic variation within genes of interest and phenotypes; 2) genome-wide association studies (GWAS). This last approach represents a powerful tool to associate phenotypic variation with the underlying sequence variants. Initially, GWAS methodology has been widely used for human genetics aimed at identifying genes involved in human disease. More recently, thanks to the rapid development of sequencing technologies and computational methods, GWAS started to be extended to crop traits studies, including rice [121], maize [122], millet [123] and sorghum [124]. Genome-wide association studies (GWAS) are computationally demanding analyses that use large sample sizes and dense marker sets to discover associations between quantitative trait variation and genetic variants. The analysis is a series of single-locus statistics tests which examine each SNP independently for the phenotype association. GWAS results depend on some factors, such as the trait heritability, genotypes number and origin, markers distribution and the association model used [125-127].

### 1.4.3 GWAS statistical methods

Several statistical models are available to identify associations between marker loci and numerous phenotypes by GWAS can reveal geneticphenotypic relationships. Nevertheless, two problems have been identified which lead this method to fail. The first problem concerns false negative marker-traits association, often attributable to small sample size and the small effect sizes expected from most QTNs. This trouble is usually overcome by increasing sample sizes. The second problem is the identification of loci that are not

responsible for the trait variation (false positives) which can be related to linkage between markers and QTNs induced by population and family structure. This challenge is met through the use of sophisticated single-marker models which consider subpopulations assignment [128], principal components of the marker matrix [129] and kinship matrices [125]. Specifically, the most effective strategies to eliminate this second problem are either fitting population structure as covariate in a General Linear Model (GLM) or fitting both population structure and kinship among individuals as covariates in a Mixed Linear Model (MLM), to make adjustments for testing markers. GLM adjusted for population structure is normally presented as y = Q + S + e, where Q (Q matrix) fitted as fixed effects stands for the proportions of individuals belonging to subpopulations (population structure), S represents the statistical tests on genetic markers, and y and e are respectively the phenotype and the residuals. MLM, adjusted both for population structure and kinship, can be written as y = Q + K + S + e, which, compared to the previous model, also introduces the kinship (K) matrix, the relationship among individuals. Compared GLM, MLM is more efficient and false positives are delated by incorporating these two factors. However, this method is computing intensive (cubic function), and, for this reason, many algorithms have been developed to reduce this computation problem. Moreover, other models have been suggested to solve the computing complexity function. With the Compressed MLM (CMLM) method, individuals are clustered into groups whose genetic value is fitted as random effect, while, with the Enriched CMLM (ECMLM) method, CMLM is enriched by integrating the group kinship. An other strategy is represented by the Factored Spectrally Transformed Linear Mixed Model (FaST-LMM), which derives kinship only on a small set of associated genetic markers (pseudo QTNs), removing the markers which are in the same region of the testing markers (within 2 Mb). With the Settlement of MLM Under Progressively Exclusive Relationship (SUPER) method, this interval is replaced with a threshold on Linkage Disequilibrium (LD) between the marker and the testing marker. Finally, with the Multi-Locus Mixed-Model (MLMM) method, differently from the previous models which conduct genetic markers tests one at a time, multiple markers are tested simultaneously tested.

FarmCPU is a GWAS method, standing for “Fixed and random modern Circulating Probability Unification”, which reflects two types of adjustments for testing genetic markers: the first one controls false positive, by fitting Q

and K as covariates; the second adjustment reduces confounding issues by either refining how K is derived from all the markers, or selectively including or excluding pseudo QTNs based on their relationship with the testing markers [130]. This model uses the MLMM which is divided into two parts: fixed effect model (FEM) and a random effect model (REM) and uses them iteratively. FEM contains testing markers, one at a time, and multiple associated markers as covariates to control false positives. To avoid model overfitting in FEM, the associated markers are estimated in REM by using them to define kinship. The P-values of testing markers and the associated markers are unified at each iteration. This model reportedly improves statistical power, increases computational efficiency, and the ability to control false positives and false negatives as compared to other models.

### 1.4.4 QTLs for mycorrhizal colonization in wheat

GWAS have already been successfully applied in wheat for many traits of interest, such as those concerning zin and copper accumulation [130], grain weight and size [131] and *Fusarium* head blight resistance [132].

Nevertheless, very few works have identified chromosomic regions associated with root AMF colonization. The first relevant has been conducted by Hetrick et al. (1995) [133] on *Triticum astivum* accessions, where, using a set of intervarietal chromosome substitution lines, have detected six different chromosomes - 1A, 5B, 6B, 7B, 5D, and 7D - which seem to be linked with mycorrhizal symbiosis. The homeologous groups 5 and 7 in the B and D genomes have showed the largest effects. More recently, Lehnert et al. (2017) [109] conducted a GWA analysis on 94 bread wheat accessions using 17823 polymorphic mapped markers. The result has revealed six QTLs - located on chromosome 3A, 4A and 7A – which have been associated to mycorrhizal colonization. Marker trait associations (MTAs) have been identified in genes associated with Chl biosynthesis, photosynthesis or sucrose cleavage, which are to be seen in the context of assimilate supply for the symbionts. 5A and 7A chromosomes were in common with that reported in the study of De Vita et al. (2018) [110], which has identified seven QTLs linked with durum wheat mycorrhizal susceptibility. Nevertheless, comparing the works, the markers linked to these two regions were located at close but different positions. This last study has found five other putative QTLs located on chromosomes 1A, 2B, 5A, 6A, 7A and 7B. Marker functional analysis has identified predicted

proteins with potential roles in hostparasite interactions, degradation of cellular proteins, homeostasis regulation, plant growth and disease/defence.

## 1.5 Aims of the thesis

The following five chapters describe the work which has been done during my PhD, with the main objective to conducte a genetic characterization on a set of tetraploid wheats and to detect chromosomal traits related to mycorrhization. Specifically, the second chapter, which represents the first paper of the thesis (published) had three objectives:

1) to characterise the population structure and the level of genetic diversity in a collection of 265 accessions of eight tetraploid *Triticum turgidum* L. subspecies, using SNPs molecular markers;
2) to reflect the evolutionary history of *Triticum turgidum* L. subspecies;
3) to explore the potential use of the SNPs markers in future phenotypic studies in order to detect genomic regions linked to quantitative traits.

The fourth chapter, which is the second paper of the thesis (published), correspond to a review summarizing the most important studies of the last 20 years, related to the identification of plants benefits due to the mycorrhizal colonization. In particular, positive advances have been reported, concerning:

1) soil nutrients uptake and traslocation;
2) resistance to abiotic stresses, including salinity, drought and heavy metals stresses;
3) resistance to pathogens.

The third chapter – the third paper of the the thesis (submitted) - illustrates a work related to the investigation of the variability and the genetic basis of susceptibility to arbuscular mycorrhizal (AM) colonization of wheat roots. The objectives of this research are:

1) to evaluate the mycorrhizal status of 130 lines of *T. turgidum* ssp. *durum*, using two AM fungal species (*Funneliformis mosseae* and *Rhizoglomus irregulare*);
2) to detect genetic markers in linkage with chromosome regions involved in AM fungal root colonization through genome wide association analyses.

The fifth chapter is a short conclusion which summarizes the results obtained in my PhD, underlining the progress and contribution given to research by this work, and its possible future developments.

The sixth and last chapter is represented by the appendix which gathers two more papers (published), which are born from two external collaborations. The first paper has been carried out in collaboration with the Università Cattolica del Sacro Cuore (Piacenza, Italy), while the second one regards my involvement in a teamwork of my university.

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# CHAPTER 2

# Molecular diversity within a Mediterranean and European panel of tetraploid wheat (*T. turgidum* subsp.) landraces and modern germplasm inferred with high-density SNPs array

## 2.1 Introduction

Wheat represents the third most important cereal grain and the most widely grown crop in the world [1]. Bread wheat (*Triticum aestivum* L.) and durum wheat (*T. turgidum* L. ssp. *durum*) are the two subspecies predominantly cultivated, used for bread-making or leavened products (cookies, cakes, and pizza) and for semolina products and pasta, respectively. In addition, both wheat species' byproducts are used for animal feed production. While bread wheat (*T. aestivum*) is hexaploid (2n = 6x = 42 chromosomes, AABBDD genomes), durum wheat belongs to the *T. turgidum* tetraploid subspecies group (2n = 4x = 28 chromosomes, AABB genomes) which includes six other subspecies (*Triticum carthlicum*, *Triticum dicoccum*, *Triticum dicoccoides*, *Triticum paleocolchicum*, *Triticum polonicum*, and *Triticum turgidum*) rarely grown commercially [2,3]. Many studies based on cytological and molecular analysis ascribe tetraploid wheat's origin to two different evolutionary steps, which started around 10,000 years ago in the Fertile Crescent [4,5]. The first divergent evolution, of which the original progenitor is unknown, gave rise to diploid species including *Triticum urartu* (A genome), *Aegilops tauschii* (D genome), *Hordeum vulgare* (barley), and Secale cereale (rye) [6]. The second evolutionary process was a natural hybridization between T. urartu (the A genome donor) and an unknown *Triticum* species, often identified as *Aegilops speltoides* (the B genome donor); this created the wild emmer *T. dicoccoides* (2n = 4x = 28, BBAA genomes), the progenitor of durum wheat [7]. The history of durum evolution is the result of domestication starting from wild emmer genotypes and of a transition process from a naked emmer type to durum type [8]. Around 7000 years Before Present (BP), durum genotypes reached the Iberian Peninsula, followed by a rapid spread from the East to the West of the Mediterranean Basin [9,10]. Natural and human selection through thousands of years led to the establishment of wheat landraces characterized by strong adaption to the environmental conditions and cultivation practices of different geographic areas [11]. Local traditional farming communities contributed to the maintenance of these landraces that were characterized by different qualitative and quantitative traits until the first decades of the twentieth century [12]. At the beginning of the 20th century, breeders imposed a strong selection based on commercial purposes: local landrace cultivation was progressively abandoned and replaced with improved, widely adapted, and more productive semi-dwarf varieties, resulting in a reduced level of genetic diversity, especially compared to the wild ancestors [13-15]. Today, this lack of diversity is widely recognized as a limiting factor in the breeding of high-

yielding and stress-resistant varieties [16]. Moreover, under the current climate change events (irregular rainfall, high temperatures during the growing season, rainstorms, and drought) that negatively affect wheat cultivation, the development of new resilient varieties or composite cross populations (CCPs) adapted to different cultivation environments and low-input agriculture has become necessary [17-19]. Novel genetic diversity selected by breeders may be introduced into modern genotypes by the introgression of useful alleles from landraces, ancestors, or wild relatives through specific breeding programs [20-22]. Durum wheat landraces and other *Turgidum* subspecies usually show a lower yield when compared to modern varieties [11]; nevertheless, they exhibit reduced productive performance compared to elite germplasm (modern varieties), but their higher genetic variability could be useful, allowing them to cope with environmental stress conditions, and to increase resilience to climate change. They are thus a potential source of favorable alleles to improve grain yield or pest resistance and to give other favorable agronomic traits to new varieties [23,24]. Recent breeding programs have studied and assessed genetic variability or different germplasm panels using different research approaches [25-32]. Morphological and agronomical markers have been considerably used [25,26], with variable reproducibility depending on environmental conditions. Nevertheless, this has been overtaken with the use of molecular markers that guarantee the opportunity of studying wheat phenotypes, providing reproducible and environment-independent results [27]. Several DNA markers have been developed and largely used to assess genetic diversity in tetraploid wheats [28- 31], but the high-density genome coverage provided in recent years by single-nucleotide polymorphism (SNP) markers has made them the best choice for wheat genetic analysis [32]. A few years ago, a novel plant breeding approach—evolutionary plant breeding (EB)—relying on human selection acting on a heterogeneous population (i.e., CCPs) started to represent a valuable method for developing populations adaptable to different agricultural contexts [33,34]. Cultivation conditions can drive the selection of more adaptable genotypes that present increased fitness [35-36]. After several years of cultivation and multiplication in the same area under isolated conditions, these populations may reach equilibrium with stable yields, and the genetic diversity among such populations represents a trait resilient to climate and environmental stress [37].

In this study, we investigated the genetic diversity and population structure of a panel of 265 accessions from seven tetraploid *T. turgidum* subspecies originating from different Mediterranean and European areas using the 35K Wheat Breeders' Axiom® SNP array. This work will prove to be a groundwork for phenotypic analysis, both in

the field and in the lab, aimed at identifying the best lines that could be used in a cross-breeding program for the selection of resilient and nutritionally improved wheat CCPs.

## 2.2 Materials and Methods

### 2.2.1 Plant Material

A large tetraploid wheat germplasm panel of 265 accessions was assembled at the Department of Agriculture (DAGRI) of the University of Florence (Supplementary Table S1). The core collection was represented by seeds of 8 *Turgidum* subspecies—ssp. *carthlicum* (5), *dicoccoides* (3), *dicoccon* (28), *durum* (172), *paleocolchicum* (3), *polonicum* (13), *turanicum* (33), and *turgidum* (7)—collected from the USDA bank (U.S. Department of Agriculture; https://npgsweb.ars-grin.gov/gringlobal/search), Wageningen CGN Germplasm bank (Centre for Genetic Resources, the Netherlands; https://www.wur.nl/en/Research-Results/Statutory-research-tasks/Centre-for-GeneticResources-the-Netherlands-1.htm) (), and Istituto di Granicoltura di Caltagirone (www.granicoltura.it). One *T. aestivum* variety—Bologna—was added to the panel as outgroup genotypes. Seeds were sown in peat-based soil in single pots and maintained in a climatic chamber at 15 °C during the night and 25 °C during the day, with a cycle of 16 h light and 8 h dark. Six weeks after germination, leaf tissue (5–6 cm section of a true leaf) was harvested from plants, immediately frozen on liquid nitrogen, and then stored at -80 °C prior to nucleic acid extraction. All plants were then transplanted in the field and grown until maturity in order to collect seeds for single-seed line constitution to be used in future field studies.

### 2.2.2 DNA extraction and genotyping

Frozen leaf tissues were ground in a TissueLyzer II bead mill (Qiagen, Hilden, Germany), with the tissue and plastic adapter having previously been dipped into liquid nitrogen to avoid sample warming. Genomic DNA was extracted from the leaf powder using a standard cetyltrimethylammonium bromide (CTAB) protocol [38] and then treated with RNase-A (New England Biolabs UK Ltd., Hitchin, UK) according to the manufacturer's instructions. DNA was checked for quality and quantity by electrophoresis on 1% agarose gel and Qubit™ fluorimetric assay

(Thermofisher), respectively. The 35K Axiom® Wheat breed Genotyping Array (Affymetrix, Santa Clara, US) was used to genotype 265 samples for 35,143 SNPs using the Affymetrix GeneTitan® system at Bristol Genomics Facility (Bristol, UK) according to the procedure described in Axiom® 2.0 Assay Manual Workflow User Guide Rev3 (https://assets.thermofisher.com/TFSAssets/LSG/manuals/702991_6-Axiom-2.0-96F-Man-WrkFlw-SPG.pdf). This array contains a range of probes that are located on chromosomes belonging to the A, B, and D genomes [39]. Since in tetraploid wheat the D genome is lacking, the effective number of markers that can be investigated is lower, corresponding to 24,240 SNPs. Allele calling was carried out using the Axiom Analysis suite software [40], and a variant call rate threshold of 92% was used instead of the default value (97%) to account for the great heterogeneity of the set analyzed [41]. The number of monomorphic and polymorphic SNP markers, the heterozygosity level, and the types of nucleotide substitution for each accession were evaluated using the same software. Monomorphic SNP markers and those with missing data points were excluded from analysis. SNP markers were then filtered for minimum allele frequency (MAF) greater than 1% and failure rate lower than 20%.

### 2.2.3 Statistical Analysis

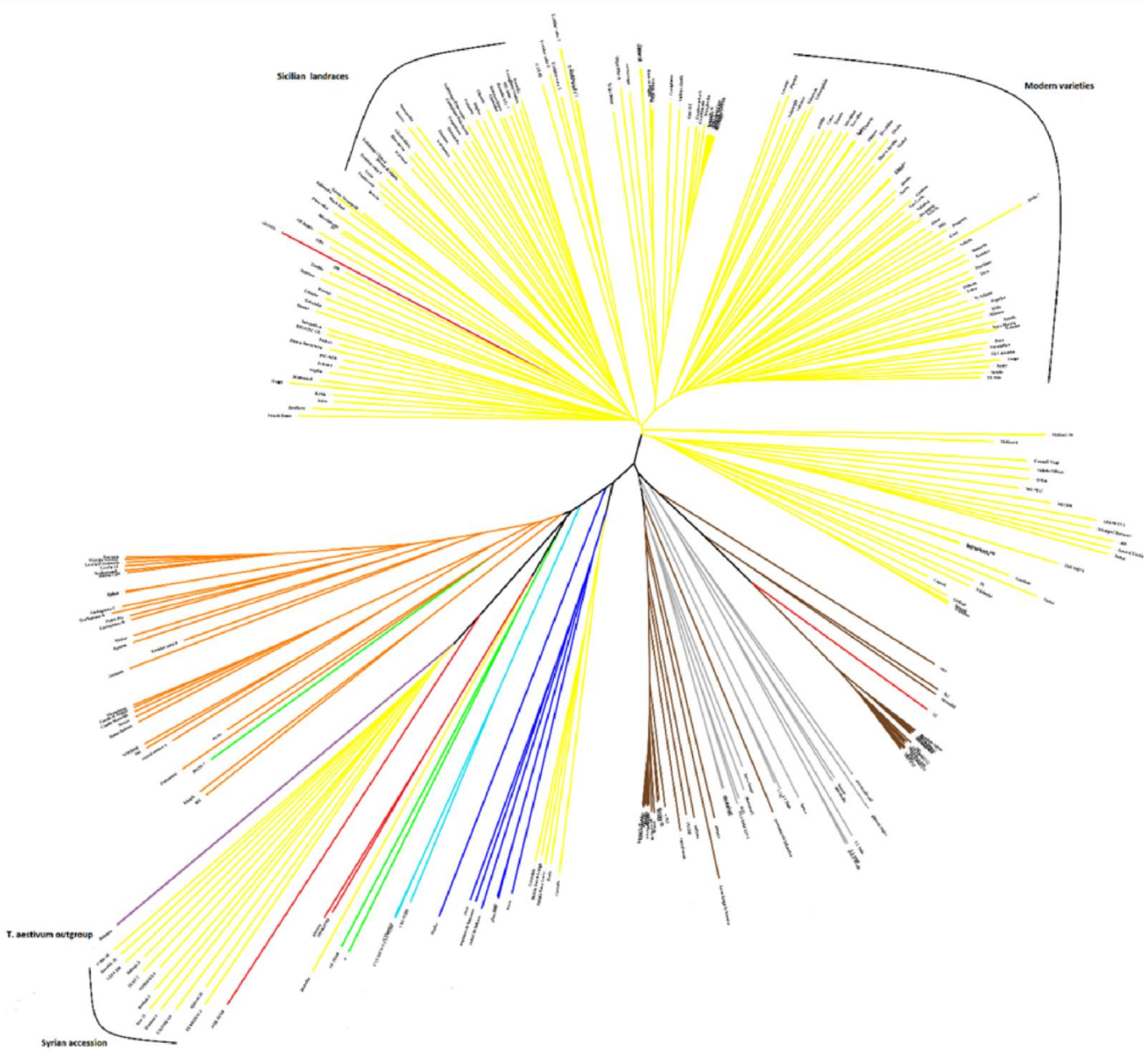
The levels and patterns of genetic diversity among accessions were investigated starting from the data obtained from SNP genotyping. The Tamura–Nei method [42] for genetic distance evaluation was applied to obtain a matrix of pairwise distances among accessions. An unrooted Bayesian tree was computed by applying the neighbor-joining algorithm [43], implemented in the *ape 3.1 package* of R software [44]. To obtain a clear picture of the genetic structure of the tetraploid wheat genotypes, we applied the Bayesian model-based clustering algorithm implemented in STRUCTURE software version 2.3.4 [45]. An admixtured and shared allele frequency model was used to determine the number of clusters (*K*), assumed to be in the range between 2 and 15, with five replicate runs for each assumed group. For each run, the initial burn-in period was set to 10,000 with 10,000 MCMC (Markov chain Monte Carlo) iterations, with no prior information on the origin of individuals. The best fit for the number of clusters, *K*, was determined using the Evanno method [46] as implemented in the program

STRUCTURE HARVESTER [47]. Structure results were then elaborated using the *R* package pophelper to align cluster assignments across replicate analyses and produce visual representations of the cluster assignments. Discriminant analysis of principal components (DAPC) was used to infer the number of clusters of genetically related individuals [48] using the *adegenet* package in R-project [49]. The first step of DAPC was data transformation using principal component analysis (PCA), while the second step was discriminant analysis performed on the retained principal components (PCs). Groups were identified using *kmeans*, a clustering algorithm that finds a given number (*k*) of groups maximizing the variation between them. k-means was run sequentially with increasing values of *k* to identify the optimal number of clusters, and different clustering solutions were compared using the Bayesian Information Criterion (BIC). The optimal clustering solution should present the lowest BIC [50].

## 2.3 Results

After SNP dataset filtering, 21,051 SNP markers were identified and used in the statistical analysis to evaluate the genetic diversity of the 265 tetraploid wheat accessions. The genetic relationships in the panel were assessed through three different approaches— neighbor-joining tree, discriminant analysis of principal components (DAPC), and STRUCTURE software—in order to better detail and define the genetic relationship variability among the tetraploid accessions. The Bayesian tree obtained by applying the neighbor-joining algorithm revealed groups in the population that highly agreed with the subspecies classification and origin (Figure 1A). Most of the *T. turgidum* ssp. *durum* (shown in yellow in Figure 1.A) were placed in a large clade together, with modern varieties that appeared separated from the other accessions. Landraces and old varieties were distributed in branches close together, mostly according to their geographical origin, such as the Syrian and Sicilian accessions. Two other clusters were identified, consisting, respectively, of *T. turgidum* spp. *dicoccon* (shown in orange) and *T. turgidum* ssp. *turgidum* (blue), while *T. turgidum* spp. *turanicum* (brown) was clustered into two groups separated by the set of *T. turgidum* ssp. *polonicum* accessions (grey). The two *T. turgidum* ssp. *paleocolchicum* accessions (light blue) and their cross seemed to be close, while the few accessions belonging to *T. turgidum carthlicum* and *dicoccoides* ssp. appeared to be spread amongst the tree branches.

**(A)**


Sicilian landraces
Modern varieties
T. aestivum outgroup
Syrian accession

**(B)**

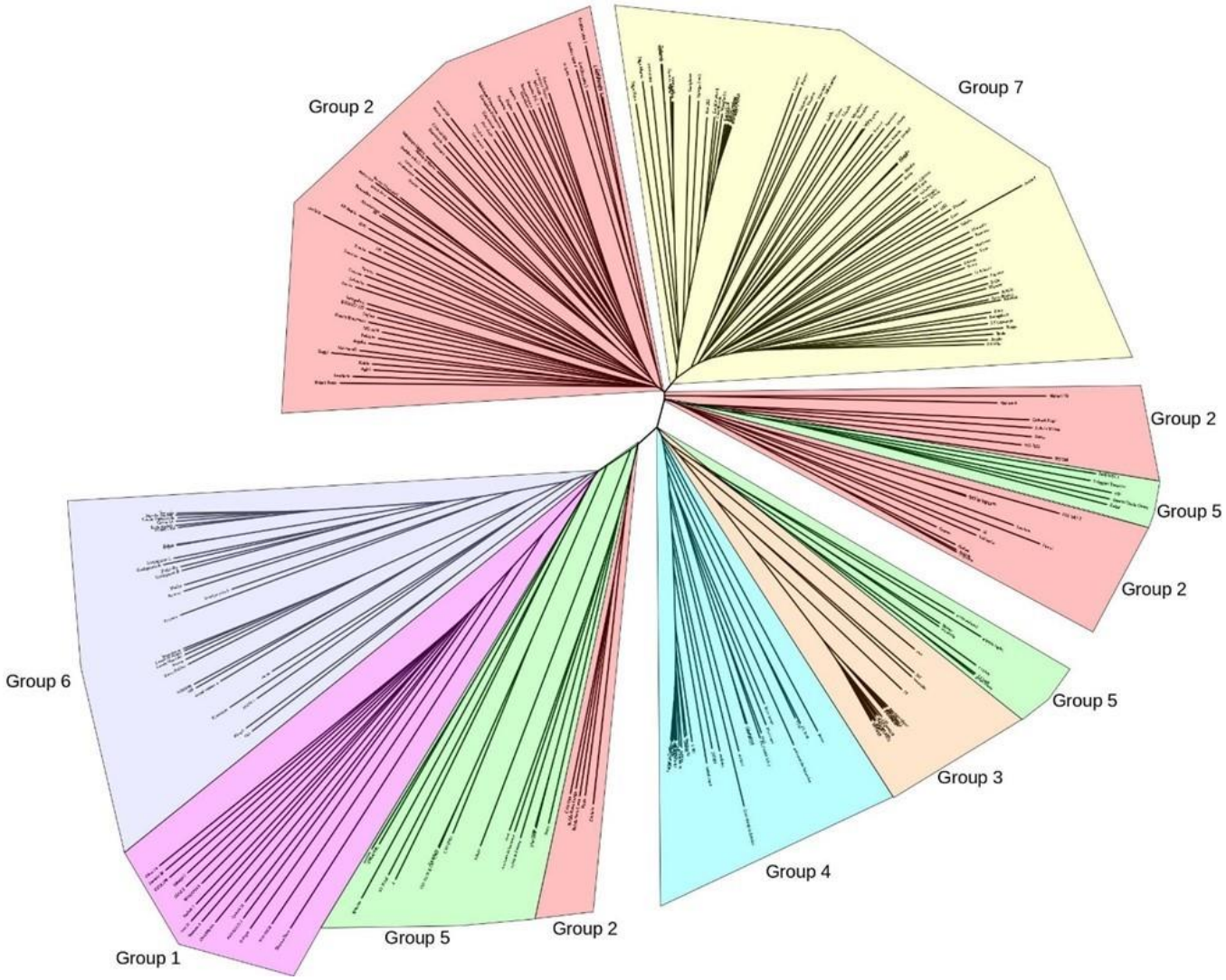



**Figure 1. (A) Bayesian tree of 265 tetraploid wheat genotypes based on single-nucleotide polymorphism (SNP) genetic markers and colored according to subspecies classification.** Branch colors: yellow for *T. turgidum* ssp. *durum*, orange for *T. turgidum* ssp. *dicoccon*, brown for *T. turgidum* ssp. *turanicum*, grey for T. turgidum ssp. polonicum, blue for T. turgidum ssp. turgidum, pale blue for T. turgidum ssp. paleocolchicum, red for T. turgidum ssp. carthlicum, green for T.turgidum dicoccoides, and violet for the T. aestivum outgroup accession. **(B) Phylogenetic tree of 265 tetraploid wheat genotypes based on SNP genetic markers and colored according to discriminant analysis of principal components (DAPC) clusterization.**

The wheat genotype arrangement obtained with the Bayesian tree was subsequently confirmed by the DAPC results (Figure 1.B, Table S2). Seven clusters (Figure 2) were detected in coincidence with the lowest Bayesian information criterion (BIC) value (Figure S1), and 100 PCs (80% of variance conserved) from PCA were retained. As

reported in Figure 1.B, the Syrian *T. turgidum* spp. *durum* wheats were pooled in Group 5 and clustered separately in the genetic tree. Most of the old varieties and landraces of the same subspecies were collected in Group 3, while Group 4 was formed by approximately half of the *T. turgidum* spp. *turanicum* accessions, which belonged to the same genetic cluster in the tree. The remaining genotypes of this last subspecies were grouped together with *T. turgidum* spp. *polonicum* wheats which were also clustered in Group 2. Group 1 was entirely composed of *T. turgidum* ssp. *diccocon* accessions, while Group 7 identified the modern varieties of *T. turgidum* ssp. *durum*. Moreover, the Bayesian tree and the DAPC analysis largely agreed with the accessions' geographic origins. In particular, Syrian (Cluster 5), French (part of the Cluster 7), Moroccan (Cluster 6), and Italian and Algerian (Cluster 3) wheats were almost entirely pooled within the same cluster. Iranian (Clusters 3 and 4) and Portuguese and American (Clusters 2 and 6) accessions were equally divided into two clusters.

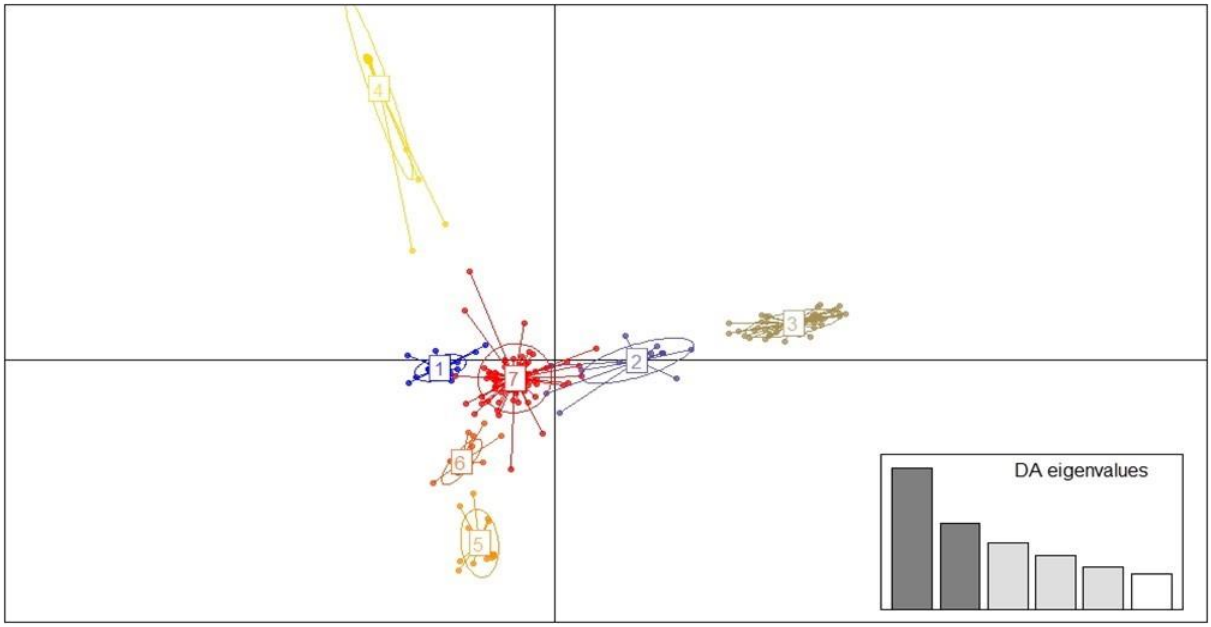



**Figure 3. Discriminant analysis of principal components (DAPC) for 264 accessions of Triticum turgidum L. used for the analysis.** The first two Linear Discriminants (LD) are represented by the axes. Each circle represents a cluster and each dot represents one accession. Numbers represent the different subpopulations identified by DAPC analysis.

The optimum number of subpopulations, *K*, estimated using STRUCTURE software (Figure 3, Table S2) and according to the Evanno method results was 7 ($K = 7$). This

indicated the presence of seven subpopulations, as previously found by the Bayesian tree and DAPC analysis, although characterized mostly by different accessions.

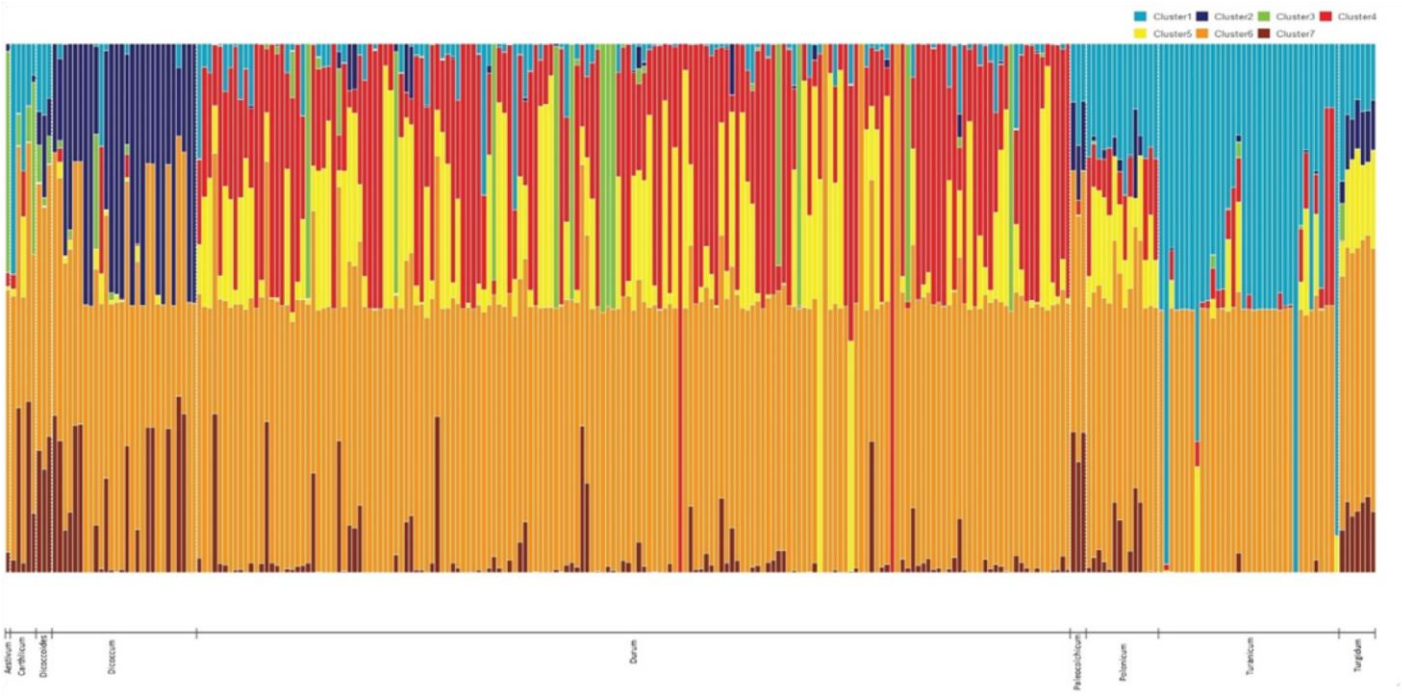



**Figure 3. Diversity in admixture analysis by STRUCTURE among 264 tetraploid wheat accessions.** Each individual is represented by a horizontal line. Color codes follow the number of clusters while the bar line under the graph represents the subspecies groups plus the outgroup genotypes (*T. aestivum*).

## 2.4 Discussion

Genetic diversity represents the basis for crop improvement, providing plant breeders with the germplasm necessary to develop cultivars with adaptive traits and good quality characteristics [51]. To better target their crossing schemes, the genetic structure and variability of 265 tetraploid wheats accessions were assessed. Clustering done via a Bayesian tree and clusters obtained via DAPC revealed a clear classification of genotypes in accordance with their geographical origin, strengthening the results of previous studies of phylogenetic relationships between cultivated wheats and their wild relatives [52,53]. Concerning *T. turgidum* ssp. *durum* accessions, which represented the largest number of genotypes in the panel, their first and second geographical origin centers—Syria and Ethiopia [54]—appeared to be clearly identified in Clusters 5 and 3, respectively. This result agreed with the molecular assessment by Kabbaj et al. [55] regarding a durum wheat

collection of cultivars. More interestingly, the Bayesian tree highlighted the proximity between North African (Morocco, Algeria, and Tunisia) and Italian germplasm; this could be linked to the geographical expansion of Romans during the Imperial Period and consequent wheat genotype introduction and cultivation on the African continent, as suggested by Rickman [56]. In addition, the positions of the accessions "Ciceredda", "Bufala rossa lunga", "Bufala nera corta", and "Paola" on the Bayesian tree deserve attention: although they belong to Cluster 3, which grouped almost all the other *T. turgidum* ssp. durum genotypes, they were gathered in a distant cluster between *T. turgidum* turgidum and polonicum ssp. The proximity of these accessions could be due to a taxonomic problem, traceable thanks to work by De Cillis [57], which classified these accessions under *T. turgidum* ssp. turgidum. Finally, another relevant observation on the *T. turgidum* ssp. *durum* accession arrangement concerns the low genetic variability detected in the modern Italian varieties, different from landraces and old varieties. Through the second half of the 20th century, national breeding programs aimed at increasing wheat yield started to establish new varieties characterized by small size, limited sprouting, reduced leaf area, and shorter crop cycle [58]. Due to genetic improvement only, De Vita et al.[59] confirmed in their work a 44% increase in productivity for the main varieties of durum wheat grown in Italy during the 20th century; however, this resulted in pure line selection and the development of varieties with low genetic variability [60]. Our study reflects this strong selection activity: Italian modern varieties were gathered in the same cluster (Figure 1.B) and along neighbor branches, highlighting genetic homogeneity. On the contrary, the subspecies *dicoccon* showed the highest genetic variability, as Laidò [61] et al. verified in their research, confirming this wild germplasm as a powerful source of genes. Today, the unpredictable climate, characterized by irregular rainfall and long dry periods, results in a rather unstable crop production. Under marginal environments, landraces and old varieties show higher stability in low-input agriculture [62,63]; thus, they could represent valuable genetic resources for breeders in order to develop new cultivars or CCP populations with specific qualitative traits such as resistance to biotic and abiotic stress, ability to efficiently use organic nitrogen and better nutritional qualities [64]. With this aim, our results showed the genetic diversity among accessions belonging to eight tetraploid wheat subspecies and identified the correct numbers of genotypes that explain the screened genetic variability well.

## 2.5 Conclusions

The genetic diversity of domesticated wheat accessions has been significantly reduced from that of their wild progenitors through a prolonged selection process for those phenotypic traits that better satisfy human needs. On the contrary, landraces' genetic variability represents a precious source of valuable agronomic traits that could be used for interspecific hybridization and for the introgression of genes and/or alleles into cultivated species. In our work, the genetic diversity and the population structure of 265 tetraploid wheats were investigated in order to understand the genetic relationships between domesticated wheats and their close wild relatives. The results obtained from this research could be used in future phenotyping studies in both field and laboratory tests to select the best lines to be intercrossed for the creation of improved and more resilient durum wheat CCP populations adapted to Mediterranean areas.

## 2.6 Supplementary materials

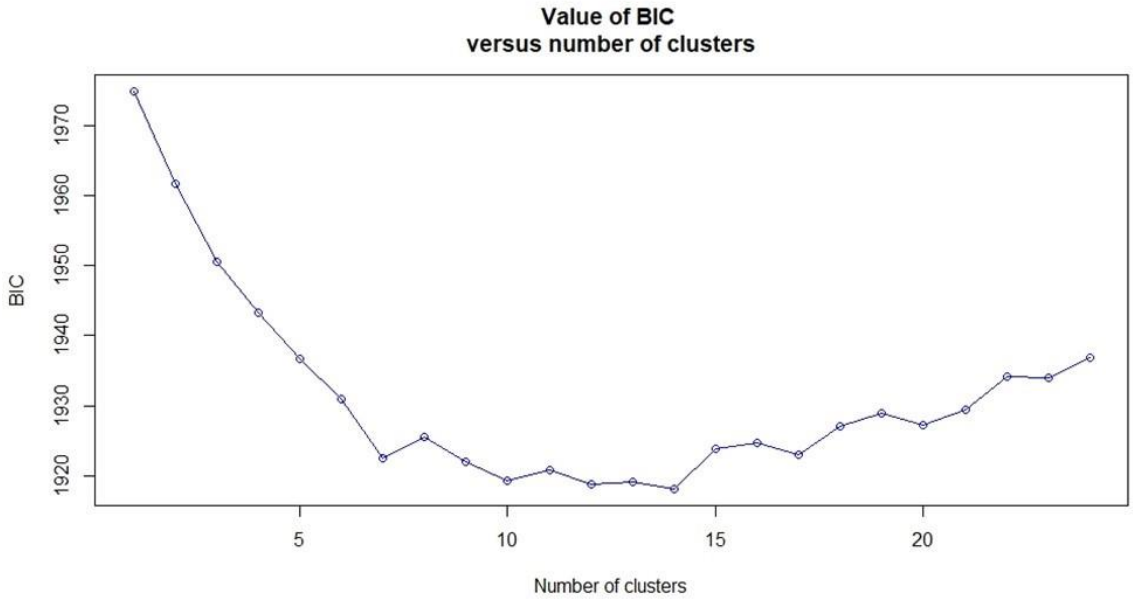



**Figure S1. Statistical determination of the optimum number of clusters by discriminant analysis of principal components (DAPC).** The elbow in the curve matches the smallest BIC, and clearly indicates that seven cluster should be retained.

**Table S1. List of wheat accessions used in the experiment**

| Accession | PI | Subspecies | Origin | Pedegree | DAPC cluster |
|---|---|---|---|---|---|
| 4 | 470944 | *T.dicoccoides* | Syria | N.D. | 5 |
| 18 | pi381966 | *T.durum* | Iran | N.D. | 2 |
| 22 | 70738 | *T.carthlicum* | Iraq | N.D. | 3 |
| 80 | clrt10125 | *T.durum* | Russian | N.D. | 2 |
| 108 | 57756 | *T.durum* | Ethiopia | N.D. | 2 |
| 145 | 225331 | *T.turanicum* | Iran | N.D. | 3 |
| 340 | CItr7779 | *T.dicoccon* | Ethiopia | N.D. | 6 |
| 362 | 68287 | *T.turanicum* | Azerbaijan | N.D. | 3 |
| 469 | 60619 | *T.durum* | Ethiopia | N.D. | 5 |
| 493 | CItr7962 | *T.dicoccon* | Ethiopia | N.D. | 6 |
| 1881 | 119341 | *T.durum* | Turkey | N.D. | 7 |
| 3823 | 190951 | *T.polonicum* | Portugal | N.D. | 4 |
| 8291 | 210852 | *T.durum* | Iran | N.D. | 2 |
| 263_a_13 | 254206 | *T.turanicum* | Iran | N.D. | 3 |
| 263_a_5 | 254198 | *T.turanicum* | Iran | N.D. | 3 |

| | | | | | |
|---|---|---|---|---|---|
| 263_a_8 | 254201 | *T.turanicum* | Iran | N.D. | 3 |
| 2N4 | 113393 | *T.turanicum* | Iraq | N.D. | 3 |
| 69Z99 7 | 355459 | *T.dicoccoides* | Armenia | N.D. | 5 |
| Abyssinicum | 352445 | *T.durum* | Ethiopia | N.D. | 2 |
| Achille | | *T.durum* | Italy | L.37/05 x AG – 4073 | 7 |
| Aethiopicum 400 | 352446 | *T.durum* | Ethiopia | N.D. | 2 |
| Agini | clrt3844 | *T.durum* | Algeria | N.D. | 2 |
| Agnone | | *T.dicoccon* | Italy | N.D. | 6 |
| AK Bugda | 572893 | *T.durum* | Azerbaijan | Apulicum 82-1/ | 2 |
| Alaska | CItr5988 | *T.turgidum* | USA | N.D. | 4 |
| Alex 283 | clrt15095 | *T.durum* | Italy | LD 390/2XS.Cappelli | 7 |
| Alzada | | *T.durum* | USA | N.D. | 7 |
| Amarai Tzada Cheru | pi191372 | *T.durum* | Ethiopia | N.D. | 5 |
| Ancomarzio | | *T.durum* | Italy | Stot//Altar84/ALD | 7 |
| Antalis | | *T.durum* | France | N.D. | 7 |
| Arcangelo | | *T.durum* | Italy | Creso x Appulo | 7 |
| Argelia | | *T.durum* | Italy | N.D. | 2 |
| Arrancada | 191823 | *T.polonicum* | Portugal | N.D. | 5 |
| Arrancada_f3 | 184543 | *T.turanicum* | Portugal | N.D. | 4 |
| Atratum | 221398 | *T.dicoccon* | Serbia | N.D. | 6 |
| Attila-7 | | *T.durum* | Mex/Syr | ND/VG9144//Kal/BB/3/Yaco/4/VEE5 | 7 |
| Aureo | | *T.durum* | Italy | Kofa/Svevo | 7 |
| AW 6629 85 | | *T.carthlicum* | Georgia | N.D. | 5 |
| Aziziah | 271894 | *T.durum* | Italy | N.D. | 2 |
| Babaga-3 | | *T.durum* | Syria | Tracha'S'//CMH76-252/PVN'S' | 1 |
| Baxter | 608017 | *T.polonicum* | USA | N.D. | 5 |
| BD1588 | 324937 | *T.durum* | Ethiopia | N.D. | 2 |
| Beloturka | CItr5014 | *T.durum* | Russian | N.D. | 2 |
| Biancu Bonorvesu | | *T.durum* | Italy | N.D. | 2 |
| Biancuccia | 278347 | *T.durum* | Italy | N.D. | 2 |
| Bidì | | *T.durum* | Italy | N.D. | 7 |
| Bishoftu | 257218 | *T.durum* | Ethiopia | N.D. | 5 |
| Black Don | 5645 | *T.durum* | Russian | N.D. | 2 |
| Bologna | | *T.aestivum* | Italy | H89092/H89136// | 1 |
| Bufala Nera Corta | 157985 | *T.durum* | Italy | N.D. | 2 |
| Bufala Rossa Lunga | | *T.durum* | Italy | N.D. | 2 |

| | | | | | |
|---|---|---|---|---|---|
| Calibasan | 166308 | *T.turanicum* | Turkey | N.D. | 4 |
| Camadi Alagi | 192130 | *T.durum* | Ethiopia | N.D. | 2 |
| Camel tooth | 321743 | *T.turanicum* | Afghanistan | N.D. | 4 |
| Capeiti | 271895 | *T.durum* | Italy | N.D. | 7 |
| Cappelli 38 | clrt12452 | *T.durum* | Italy | N.D. | 7 |
| Casale de litappi | | *T.dicoccon* | Italy | N.D. | 6 |
| Casale macirillo | | *T.dicoccon* | Italy | N.D. | 6 |
| Cascia 14 | | *T.dicoccon* | Italy | N.D. | 6 |
| Cascia primaverile | | *T.dicoccon* | Italy | N.D. | 6 |
| Castel fusano | 347730 | *T.durum* | Italy | selection from mutagenized | 7 |
| Castelporziano | 347731 | *T.durum* | Italy | selection from mutagenized | 7 |
| Castiglione Glabro | 157974 | *T.durum* | Italy | N.D. | 2 |
| Castiglione Pubescente | 157981 | *T.durum* | Italy | N.D. | 2 |
| Chamran/4/cmh82a.1294 | | *T.durum* | Syria | | 1 |
| Chiattulidda | 157962 | *T.durum* | Italy | N.D. | 2 |
| Ciciredda | | *T.durum* | Italy | N.D. | 2 |
| Civitella | 231363 | *T.durum* | Italy | N.D. | 5 |
| Claudio | | *T.durum* | Italy | Sel. Cimmyt/Durango//IS1938/Grazia | 7 |
| clrt 7656 | | *T.carthlicum* | | N.D. | 2 |
| Core | | *T.durum* | Italy | N.D. | 7 |
| Cotrone | 294571 | *T.durum* | Italy | N.D. | 2 |
| Creso | | *T.durum* | Italy | Cpb 144 x [(Yt54-N10-B) Cp2 63 Tc1 ] | 7 |
| Dabat | 192115 | *T.durum* | Ethiopia | N.D. | 5 |
| Dauno | 174619 | *T.durum* | Italy | N.D. | 2 |
| Dauno Strampelli | 191504 | *T.durum* | Italy | N.D. | 2 |
| Daurur | | *T.durum* | France | N.D. | 7 |
| Dendan_fil | 337643 | *T.turanicum* | Afghanistan | N.D. | 4 |
| Dur de Varna | 174628 | *T.durum* | Italy | N.D. | 2 |
| Duro de Naples | 192525 | *T.durum* | Italy | N.D. | 7 |
| Duro di Puglia | | *T.durum* | Italy | N.D. | 7 |
| E_707 | 283795 | *T.turanicum* | Afghanistan | N.D. | 4 |
| ELS 6304-1 | Cltr14750 | *T.dicoccon* | Ethiopia | N.D | 4 |
| etrusco_1 | | *T.turanicum* | Italy | N.D. | 4 |
| Ettore | | *T.durum* | Italy | Selection.Cimmyt/Durango// IS1938 | 7 |
| Evoldur selection1 | | *T.durum* | Italy | N.D. | 4 |
| Evoldur selection2 | | *T.durum* | Italy | N.D. | 4 |
| Evoldur selection3 | | *T.durum* | Italy | N.D. | 2 |

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|---|---|---|---|---|---|
| Evoldur selection4 | | *T.durum* | Italy | N.D. | 2 |
| Evoldur selection5 | | *T.durum* | Italy | N.D. | 2 |
| Evoldur selection6 | | *T.durum* | Italy | N.D. | 7 |
| Evoldur selection8 | | *T.durum* | Italy | N.D. | 6 |
| Farro lungo | 278350 | *T.dicoccon* | Italy | N.D. | 4 |
| Farro lungo la fiandra | | *T.turanicum* | Italy | N.D. | 4 |
| Felasit Rosso | 192035 | *T.durum* | Ethiopia | N.D. | 2 |
| Ferroug-3/qafzah-33 | | *T.durum* | Syria | N.D. | 1 |
| Fiorentino | | *T.dicoccon* | Italy | N.D. | 6 |
| Francesa | 157954 | *T.durum* | Italy | N.D. | 2 |
| Francesone | 157955 | *T.durum* | Italy | N.D. | 2 |
| Fuego | | *T.durum* | Italy | N.D. | 7 |
| G 51/48 | 209271 | *T.durum* | Australia | Aleppo/Kubanca | 2 |
| Gaggi | 192421 | *T.durum* | Ethiopia | N.D. | 2 |
| Gandum | 142521 | *T.durum* | Iran | N.D. | 2 |
| Garfagnana a | | *T.dicoccon* | Italy | N.D. | 6 |
| Garfagnana b | | *T.dicoccon* | Italy | N.D. | 6 |
| Garfagnana c | | *T.dicoccon* | Italy | N.D. | 6 |
| Garigliano | 352417 | *T.durum* | Italy | N.D. | 7 |
| Giemme | | *T.durum* | Italy | Duilio x Grazia | 7 |
| Gigante | 157956 | *T.durum* | Italy | N.D. | 2 |
| Gigante inglese | 184526 | *T.turanicum* | Portugal | N.D. | 5 |
| Girgentana | 157963 | *T.durum* | Italy | N.D. | 2 |
| Giustalisa | 157970 | *T.durum* | Italy | N.D. | 2 |
| Giza-168/Shuha's'/dobuc's' | | *T.durum* | Syria | N.D. | 1 |
| Gondum_ishtur | 317492 | *T.turanicum* | Afghanistan | N.D. | 3 |
| Grazia | | *T.durum* | Italy | M 6800 127 x VALSEVA | 7 |
| graziella_ra | 623656 | *T.turanicum* | Italy | N.D. | 4 |
| Grifoni | 271898 | *T.durum* | Italy | VU/S.Cappelli | 2 |
| Hamam-4 | | *T.durum* | Syria | T.aest/SPRW'S'//CA8055/3/Bacanora86 | 1 |
| Heraklion | | *T.durum* | Italy | N.D. | 7 |
| Husco | 286547 | *T.polonicum* | Ecuador | N.D. | 2 |
| I_1_3490 | 272570 | *T.polonicum* | Hungary | N.D. | 5 |
| I_1_3496 | 272564 | *T.polonicum* | Hungary | N.D. | 5 |
| I-1-3448 | 272590 | *T.polonicum* | Hungary | N.D. | 4 |
| Iar/w/51-1 | 387391 | *T.durum* | Ethiopia | N.D. | 5 |
| Iar/W/67-3 | 387434 | *T.durum* | Ethiopia | N.D. | 2 |

| | | | | | |
|---|---|---|---|---|---|
| Inglesa | | *T.durum* | Italy | N.D. | 2 |
| Iride | | *T.durum* | Italy | Altar84/Ionio | 7 |
| IWA8606551 | 623656 | *T.turanicum* | Azerbaijan | N.D. | 3 |
| IWA8607904 | 624217 | *T.turanicum* | Iran | N.D. | 3 |
| IWA8608332 | 624429 | *T.turanicum* | Iran | N.D. | 3 |
| IWA860984 | 625155 | *T.turanicum* | Iran | N.D. | 3 |
| IWA8609932 | 625164 | *T.turanicum* | Iran | N.D. | 3 |
| Izaz-11 | | *T.durum* | Syria | Shuha-7/4/NIF/3/Soty//NAD63/Chiris | 1 |
| Izaz-2/asfoor-1/4/opata/bow// | T.durum | *T.durum* | Syria | N.D. | 1 |
| Jawahir-20 | | *T.durum* | Syria | Shuha-4//NS732/Her | 1 |
| Kahla | 7794 | *T.durum* | Algeria | N.D. | 2 |
| Kamut | | *T.turanicum* | North Africa | N.D. | 4 |
| Kamut porfiri | | *T.turanicum* | Italy | N.D. | 4 |
| Kanakis | | *T.durum* | France | N.D. | 7 |
| Levante | | *T.durum* | Italy | G80/Piceno//Ionio | 7 |
| Leventis | 306665 | *T.turanicum* | France | N.D. | 4 |
| Mahmoudi | Cltr3809 | *T.durum* | Tunisia | N.D. | 2 |
| Maliani 1D | 322735 | *T.durum* | Italy | N.D. | 2 |
| Maliani 6 | 324936 | *T.durum* | Italy | Russello/Forlani | 2 |
| Manto di Maria | | *T.durum* | Italy | N.D. | 2 |
| Marche Simona | | *T.dicoccon* | Italy | N.D. | 6 |
| Marco Aurelio | | *T.durum* | Italy | Orobel//Arcobaleno/Svevo | 7 |
| Margherito | | *T.durum* | Italy | N.D. | 7 |
| Marrocos | 191599 | *T.turanicum* | Morocco | N.D. | 4 |
| Martinari | 134945 | *T.polonicum* | Portugal | N.D. | 4 |
| Martinella | 157971 | *T.durum* | Italy | N.D. | 2 |
| Martinur | | *T.durum* | France | N.D. | 7 |
| Meknes | 192658 | *T.turanicum* | Morocco | N.D. | 4 |
| MG 4479 | 470769 | *T.durum* | Italy | N.D. | 2 |
| MG 4483 | 470773 | *T.durum* | Italy | N.D. | 7 |
| MG 4486 | 470776 | *T.durum* | Italy | N.D. | 2 |
| MG 7852 | 470785 | *T.durum* | Ethiopia | N.D. | 2 |
| Mimmo | | *T.durum* | Italy | SimetoXMedora | 7 |
| Mirabella | 352488 | *T.polonicum* | Italy | N.D. | 5 |
| Miradoux | | *T.durum* | France | N.D. | 7 |
| Molise | | *T.dicoccon* | Italy | N.D. | 6 |

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|---|---|---|---|---|---|
| Molise colli | | *T.dicoccon* | Italy | N.D. | 6 |
| Monastir | | *T.durum* | France | N.D. | 7 |
| Monteleone | | *T.dicoccon* | Italy | N.D. | 6 |
| Nonette de lausanne | 352544 | *T.turgidum* | Switzerland | N.D. | 5 |
| Norcia | | *T.dicoccon* | Italy | N.D. | 6 |
| Novo | 221423 | *T.turgidum* | Portugal | N.D. | 5 |
| Obelix | | *T.durum* | Italy | N.D. | 7 |
| Odisseo | | *T.durum* | Italy | N.D. | 7 |
| Oeltta | 192064 | *T.durum* | Ethiopia | N.D. | 2 |
| Orobel | | *T.durum* | France | Composite INRA | 7 |
| Padre pio | | *T.dicoccon* | Italy | N.D. | 6 |
| Paola | | *T.durum* | Italy | N.D. | 2 |
| Paprika | | *T.durum* | France | N.D. | 7 |
| Pavone | 157977 | *T.durum* | Italy | N.D. | 2 |
| Pelissier | 7785 | *T.durum* | Algeria | N.D. | 2 |
| Perciasacchi | | *T.turanicum* | Italy | selection from | 4 |
| Pererodka | 2954 | *T.durum* | Russian | N.D. | 2 |
| Persian | 283888 | *T.carthlicum* | Iran | N.D. | 5 |
| Pharaons | 352543 | *T.turgidum* | France | N.D. | 5 |
| PI_369816 | 369816 | *T.paleocolchicum* | Russian | N.D. | 5 |
| Pigreco | | *T.durum* | Italy | N.D. | 7 |
| Polsk_Hvede | 361757 | *T.polonicum* | Denmark | N.D. | 5 |
| Preziusa | | *T.durum* | Italy | N.D. | 7 |
| Prospero | | *T.durum* | France | N.D. | 7 |
| Puro | | *T.durum* | Italy | N.D. | 7 |
| Qafzah-31 | | *T.durum* | Mex/syr | Sha5//Carc/Auk/3/Vee5//Doubc'S' | 1 |
| Radicondoli | | *T.dicoccon* | Italy | N.D. | 6 |
| Ramirez | | *T.durum* | France | N.D. | 7 |
| Reale | | *T.durum* | Italy | N.D. | 7 |
| Realforte | 41038 | *T.durum* | Tunisia | N.D. | 2 |
| Reehab-2 | | *T.durum* | Syr | Vee/Koel//Attila-5 | 1 |
| Regina | 264956 | *T.durum* | Italy | N.D. | 2 |
| RIGA 952/20135 | Cgn08251 | *T.durum* | Italy | N.D. | 2 |
| Rivet | 278221 | *T.turgidum* | UK | N.D. | 5 |
| Roseta | 192399 | *T.durum* | Italy | N.D. | 2 |
| Rosso rubino | | *T.dicoccon* | Italy | N.D. | 6 |
| Rubiel_de_liebana | 191203 | *T.turgidum* | Spain | N.D. | 5 |

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|---|---|---|---|---|---|
| Ruscia | 264957 | *T.durum* | Italy | N.D. | 2 |
| Russello | 157973 | *T.durum* | Italy | N.D. | 2 |
| Russello S.G. 7 | 231357 | *T.durum* | Italy | N.D. | 2 |
| Sabaudia | 352418 | *T.durum* | Italy | N.D. | 2 |
| Sammartinara | 157958 | *T.durum* | Italy | N.D. | 2 |
| San Carlo | | *T.durum* | Italy | GraziaXDegamit | 7 |
| Saragolla | | *T.durum* | Italy | Iride/Linea PSB 0114 | 7 |
| Saragolla | 231380 | *T.durum* | Italy | N.D. | 7 |
| Sari_tuya_tish | 290530 | *T.turanicum* | Hungary | N.D. | 3 |
| Scavuzza | 157966 | *T.durum* | Italy | N.D. | 2 |
| Sciaggari Busasore | 192031 | *T.durum* | Ethiopia | N.D. | 5 |
| Scorsonera | 264955 | *T.durum* | Italy | N.D. | 2 |
| Secolo | | *T.durum* | Italy | N.D. | 7 |
| Semenzella | 157972 | *T.durum* | Italy | N.D. | 2 |
| Senatore Cappelli | 352414 | *T.durum* | Italy | N.D. | 7 |
| Sert | 167481 | *T.turanicum* | Turkey | N.D. | 2 |
| Sin_el_pheel | 208911 | *T.polonicum* | Iraq | N.D. | 4 |
| Solex | | *T.durum* | Italy | CresoXValgerardo | 7 |
| Somama-<br>9//seri82/shuha's' | T.durum | *T.durum* | Syria | | 1 |
| Suhulu Villoso | 192743 | *T.durum* | Ethiopia | N.D. | 2 |
| Sunray | CItr11390 | *T.turanicum* | United<br>States | United States | 4 |
| Svevo | | *T.durum* | Italy | Cimmyt landreces/Zenit | 7 |
| Svevo x Senatore Cappelli | | *T.durum* | Italy | SvevoXS.Capelli | 7 |
| SY 20198 | 487264 | *T.dicoccoides* | Syria | N.D. | 5 |
| Sy Atlante | | *T.durum* | Italy | N.D. | 7 |
| SY Esperto | | *T.durum* | Italy | N.D. | 7 |
| SY Leonardo | | *T.durum* | Italy | N.D. | 7 |
| SY Nilo | | *T.durum* | Italy | N.D. | 7 |
| T_742 | 352487 | *T.polonicum* | Germany | N.D. | 4 |
| Tafeelih riti | 223171 | *T.polonicum* | Jordan | N.D. | 4 |
| Taganrog | CItr3979 | *T.durum* | Russian | N.D. | 7 |
| Tanis | 352542 | *T.turgidum* | France | N.D. | 5 |
| Tedone 10 | | *T.turanicum* | Italy | N.D. | 7 |
| Tedone 8 | | *T.turanicum* | Italy | N.D. | 7 |
| Timilia | 278356 | *T.durum* | Italy | N.D. | 2 |
| Tirex | | *T.durum* | Italy | SvevoXNefer | 7 |

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|---|---|---|---|---|---|
| Tiruni | 151919 | *T.durum* | Iran | N.D. | 2 |
| Toscadou | | *T.durum* | France | N.D. | 7 |
| Trentino | 157965 | *T.durum* | Italy | selection from landrace from Caltanissetta | 2 |
| Trigu Biancu | | *T.durum* | Italy | N.D. | 7 |
| Trigu Moro | | *T.durum* | Italy | N.D. | 2 |
| Trigu Murru | | *T.durum* | Italy | N.D. | 7 |
| Trionfo | | *T.durum* | Italy | N.D. | 7 |
| Tripolino | 157959 | *T.durum* | Italy | N.D. | 2 |
| Tunisina | 157967 | *T.durum* | Italy | N.D. | 2 |
| Turnadili | 166959 | *T.turanicum* | Turkey | N.D. | 3 |
| Tuscania | | *T.dicoccon* | Italy | N.D. | 6 |
| Urria | 157968 | *T.durum* | Italy | Turkey | 2 |
| Usher-18 | | *T.durum* | Syria | CROW'S'/BOW'S'-1994/ | 1 |
| Vakilagha | 210382 | *T.durum* | Iran | N.D. | 2 |
| Valaniene | Clrt15147 | *T.durum* | Italy | Cappelli/V.Z. 156 | 7 |
| Valerio | | *T.durum* | Italy | Canadian landreces/ | 7 |
| Valgiorgio | Cltr15217 | *T.durum* | Italy | Cappelli/V.Z. 156 | 7 |
| Vallega Zitelli | Cltr15096 | *T.durum* | Italy | Cappelli/Yuma | 7 |
| Vallelunga Glabra | | *T.durum* | Italy | N.D. | 2 |
| Vallelunga Pubescente | 157980 | *T.durum* | Italy | N.D. | 2 |
| Valsacco | 367229 | *T.durum* | Italy | Cappelli/VZ 156 | 7 |
| Valselva | 367212 | *T.durum* | Italy | Giorgio 324// | 7 |
| Valtarquinio | 367225 | *T.durum* | Italy | Cappelli/VZ 156 | 7 |
| Vernal emmer | Clrt3686 | *T.dicoccon* | United States | N.D. | 5 |
| WIR 6388 | 349043 | *T.dicoccon* | Georgia | N.D. | 6 |
| WIR28162 | 349050 | *T.paleocolchicum* | Georgia | N.D. | 5 |
| WIR28162XPI_369816 | | *T.paleocolchicum* | | N.D. | 5 |
| WIR32510 | 341800 | *T.carthlicum* | Russian | N.D. | 1 |
| Yaqub | | *T.dicoccon* | Italy | N.D. | 6 |

| | | | | | |
|---|---|---|---|---|---|
| Zefiro | | *T.dicoccon* | Italy | N.D. | 6 |
| Zetae | | *T.dicoccon* | Italy | N.D. | 7 |

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# CHAPTER 3

## Studies from the last twenty years on plant–arbuscular mycorrhizal fungi associations and their uses for wheat crops

## 3.1 Introduction

A mycorrhiza (from Greek, mykos: fungus and Rhiza: root) is a particular symbiotic association between a higher plant and a fungus that is located in the plant root apparatus and extends into the rhizosphere and the surrounding soil. This symbiosis, named "mycorrhiza" by Frank in 1885 [1], has become a very interesting research object for the agronomic sector, with particular attention to its positive effects on the most important crops, mainly thanks to the studies of Peyronel in Italy, Melin in Sweden, and Harley in England [2]. Nowadays, we are aware that besides being present in almost all plants in good vegetative conditions [3], mycorrhizae represent a form of "biological fertilization" [4], with the results being very effective for plants, especially in conditions of abiotic or biotic stresses. According to the different associations between plant and fungus, mycorrhizae are classified into three groups: ectomycorrhiza, endomycorrhiza, and ectoendomycorrhizae (with mixed behavior between ectomycorrhiza and endomycorrhizae) [2]. Recently, research attention has been focused mostly on the second group. Ectomycorrhiza are formed by *Basidiomycetes* and *Ascomycetes* fungi with the roots of plants from cold temperate regions, including firs, pines, and larches in borealis and subalpine forests, and oaks and poplars in deciduous forests [5]. The fungus, which is an obligatory symbiont, forms a mantle of hyphae around the root and completely envelops the root tip but never enters the inside of the cells [6]. Endomycorrhiza differ from the previous type in structure: the hyphae of the fungus not only grow inside the root of the plant, but penetrate the root cell walls and become enclosed in the cell membrane as well [7]. This makes for a more invasive symbiotic relationship between the fungus and the plant. Endomycorrhiza have further been classified into five major subgroups — arbuscular, ericoid, arbutoid, monotropoid, and orchid mycorrhizae [8] — but arbuscular mycorrhizae (AM), which are the most ubiquitous in nature and affect many plants (both cultivated and living in natural ecosystems), are the most debated and studied in agriculture research [9]. The fungi developing into AM belong to the phylum *Glomeromycota* and form mutualistic relationships with over 80% of all vascular plants [10]; once in contact with the host's root, they penetrate through the epidermis and establish their hyphae in the cortical parenchyma [11]. The penetration of the fungus inside the root surface can take place via three different modalities: it can form an appressorium, from which intracellular hyphae originate; it can penetrate through a radical hair; or it can enter across the cells of external layers that

are often dead and flake off [2]. At this point, the hyphae, deepening in the root, abundantly colonize the cells and undergo intense branching, forming arbuscules with a life cycle of 7–12 days [12], which act as the site for fungus/plant metabolite exchange and for the accumulation of reserve nutrients [13]. The fungus does not come into contact with the cytoplasm of the host plant but is always separated from the host cell plasmalemma that is absorbed during the penetration and involved in all developmental stages of the symbiotic interaction [14] (Figure 1). These fungi are obligate symbionts and form a mutualistic symbiotic association with the plant through an exchange of substances between them: the fungus receives carbon to complete its life cycle and, at the same time, it provides nutritional benefits to the plant [15]. Of the carbohydrates produced by the plant through photosynthesis, 10% to 40% can be absorbed by mycorrhizal fungi [16]. Moreover, extraradical mycelial networks seem to maintain their ability to establish mycorrhizal symbioses with plants as long as five months after shoot removal, adsorbing nutrients released from dead roots [17].

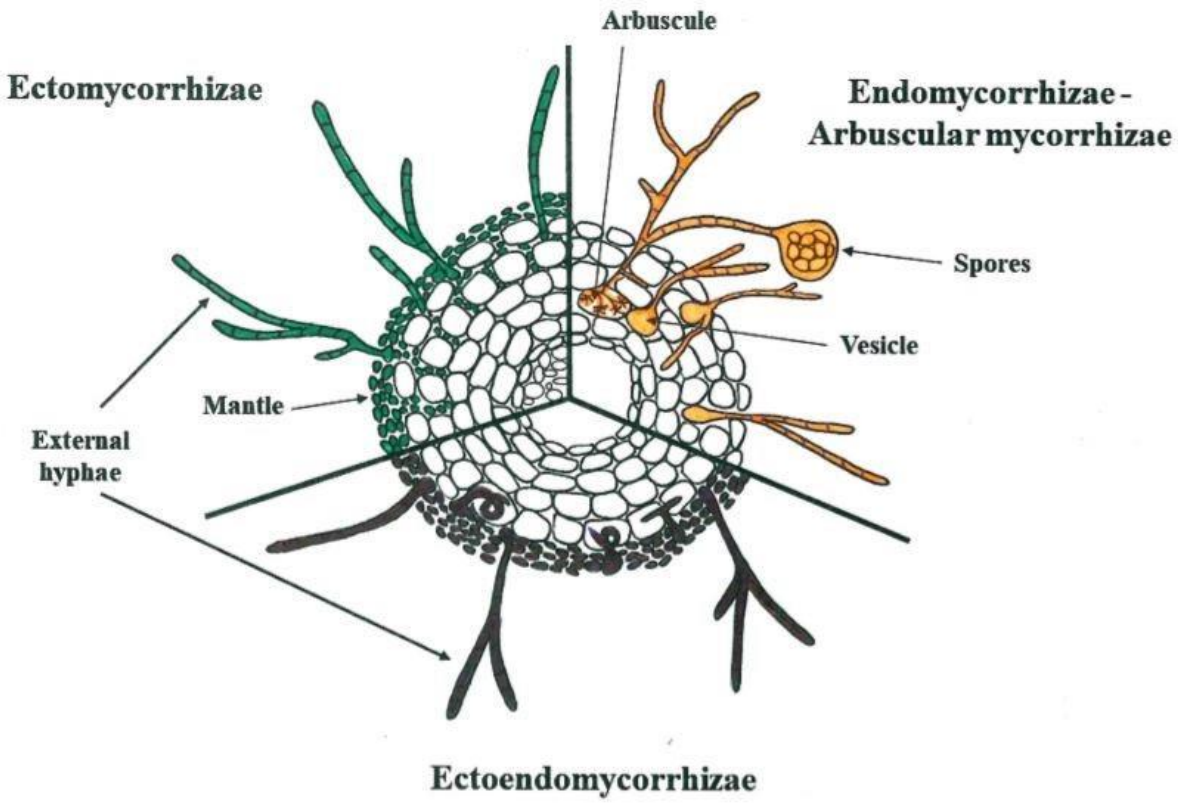


**Fig. 1. Different associations between a mycorrhizal fungus and plant roots.** Arbuscular mycorrhizal fungi (on the right) penetrate the cortical cells of the root, forming structures such as arbuscules and vesicles. Ectomycorrhizal fungi (on the left) completely cover the plant root system with a mantle of fungal tissue, and the hyphae surround the plant cells within the root cortex. With ectoendomycorrhizal fungi (bottom), the fungus mantle is formed but the hyphae may also penetrate the plant cells.

Currently, many scientific works have involved multidisciplinary approaches to understanding arbuscular mycorrhizal fungi (AMF) symbiotic association, underlining the importance of their use in sustainable and organic agriculture in relation to the most cultivated crops. The notable relevance of wheat in the Mediterranean human diet, with total production of 756 million tons for 2017–2018 (FAO 2018), has motivated and encouraged research towards the experimental use of mycorrhizal fungi in order to guarantee the better performance of plants. This direction has two principal goals: firstly, a lower dependence on chemical fertilizers and pesticides, and secondly, crop protection from abiotic stress (lack of water, salinity, excess phytotoxic elements).

## 3.2 Uptake and translocation of soil nutrients

Many studies have examined the key role of arbuscular mycorrhizal fungi (AMF) in nutrient uptake and translocation and discovered a plant root surface area increment. First, the ability of mycorrhizal hyphae to extend and explore a greater area of soil than the host plant's roots can reach has been demonstrated [18]. The fungus seems to be a great help in the acquisition of soil nutrients, reaching micro and macro elements which the plant alone could not reach. In this way, the depletion zone created via a plant's rapid nutrient uptake in the proximity of its root system [19] can be bridged, and an adequate supply of nutrient elements is translocated to the plant [20]. Secondly, fungal hyphae dimensions positively influence nutrient uptake: their narrow diameter (2–20 μm) allows them to access small pores that are unreachable for plant roots [21], with important implications in terms of water and micronutrient absorption [22]. In this context, Drew et al. [23] evidenced a further mycorrhizal ability to adjust the hyphal diameter depending on the soil pore size and, in this way, provide for plant nutrition independently of soil texture. Some other studies have linked the increased plant acquisition of mineral nutrients to AMF production of some organic acids and enzymes which represent, respectively, a source of phosphorus and a means to depolymerize organic nitrogenous polymers and to take up nitrogen from secondary sources of organic matter like dead microbial biomass [24,25]. The positive effect of mycorrhizae on wheat plants' response to nutrient uptake has been recently well documented by many researchers. A meta-analysis conducted on 38 published field trials with 333 observations highlighted the beneficial effect of field AMF inoculation on wheat dry weight and P, N, and Zn

uptake [26]. Concerning zinc (Zn) uptake, AMF contribution was tested by Ma et al. [27], who revealed a Zn concentration 1.13–

2.76 times higher in wheat plants treated with *Funneliformis mosseae* and *Rhizophagus intraradices*, and by Ryan and Angus [28], who showed an increase of Zn absorption with *Rhizophagus irregularis* and *Scutellospora calospora* administration to the plant. Moreover, a positive correlation between grain Zn concentration and the level of colonization by AMF was suggested in a study by Ryan et al. [29]. Advantageous results for phosphorus (P) nutrition were found with *Rhizophagus irregularis* inoculum and spring wheat (*Triticum aestivum*), supporting a mycorrhizal fungi contribution of more than 50% of the P absorption by the plant [30], and with *Funneliformis mosseae* and durum wheat (*Triticum durum*, cv. Petra), showing a plant dependency on mycorrhizae for P uptake [31]. High differences in P plant acquisition were also obtained in *Claroideoglomus etunicatum* inoculated plants (*Triticum aestivum*, cv. Otto), reflecting the beneficial mineralizing phosphatase (P-ase) effect of the AM-fungus-colonized roots. The first evidence of the positive interactions among AMF and bacteria isolated from their spores was provided by Battini et al. [32], who showed increased P uptake via a mycorrhizal pathway in maize plants with a particularly efficient strain, *Streptomyces* sp. In field conditions, a synergistic effect of plant-growthpromoting rhizobacteria and AMF on wheat P uptake was observed: a higher P content (67.8 mg plant 1 ) was observed with the co-inoculation of *Azotobacter chroococcum* with *Bacillus* spp. and *Rhizophagus fasciculatus* [33]. Enhanced selenium (Se) content in wheat grain through inoculation with AM fungi was documented by Luo et al. [34], who showed, after inoculation with *Diversispora epigaea* (formerly *Glomus versiforme*) or *Funneliformis mosseae*, a significant increase in selenate and selenite uptake by the wheat roots in hydroponic experiments. Positive results for Se uptake were also found in an experiment on wheat (*Triticum aestivum*, cv. Otto) by Durán et al. [35], where applications of selenobacteria strains (*Stenotrophomonas* sp. B19, *Enterobacter* sp. B16, *Bacillus* sp. R12 and *Pseudomonas* sp. R8) with the AM fungus *Glomus claroideum* resulted in a 23.5% higher (725 mg kg 1 ) selenium content than that in non-mycorrhizal plants (587 mg kg 1 ). A recent study by Blackwell et al. [36] investigated inoculation with AMF combined with biochar treatment to improve wheat mineral nutrition. This research showed how biochar–mineral complexes (BMCs), coupled with the AM fungus, increase the nutrient uptake of wheat (*Triticum aestivum*, cv. Wyalkatchem), particularly for N, P, K, S, and Zn. These

results confirm Saito and Marumoto's [37] explanation that biochar influences microbial activity by providing a weakly alkaline microhabitat within the pore spaces.

## 3.3 Abiotic stresses

Several studies on abiotic stresses have shown how human activities connected to agriculture (excessive use of pesticides and fertilizers, irrigation) and construction (deforestation, waste material diffusion) have negatively affected plant growth, health, and yield, causing significant and catastrophic production losses [38,39]. Plant responses to stresses involve a generic pathway that starts from stress signal acquisition by the membrane receptor and ends with the expression of genes, the products of which can provide plant defense, directly or indirectly [40]. Nevertheless, many studies on AM symbiosis have confirmed the contribution of the involved fungus in helping the plant to resist abiotic stress—such as drought, salinity, and heavy metal contamination—through the implementation of various mechanisms.

### 3.3.1 Salinity stress

High salt concentrations in the soil make it harder for roots to extract water and can be toxic to plant functions [41], resulting in some negative outcomes, such as ethylene production, plasmolysis, nutrient imbalance, interference with photosynthesis, and the production of reactive oxygen species (ROS) [42]. Osmotic adaptation, represented by the accumulation of compatible solutes (such as proline and glycine betaine) within the plant cells, represents a physiological mechanism for the plant that allows it to maintain a gradient of water potential between the cells without a decrease in turgor or growth [43]. However, many recent scientific works have evidenced the existence of salttolerant AMF species that, being able to maintain good efficiency in root colonization and symbiosis formation, help the plants to overcome the salinity problem. In particular, scientists refer to four different AMF abilities to alleviate salt stress, which are the following:

(1) Higher water uptake: mycorrhizal hyphae can better expand into the soil, taking up more water and preventing plant dehydration and turgor loss—two consequences of salinity compromising the water status of the plant [44];

(2) Increased mineral nutrition linked to maintaining a high K/Na ratio [45]: under osmotic stress conditions, the soil Na+ concentration is very high and negatively interferes with other various transporters in the root plasma membrane, such as K+ selective ion channels [46]. As a consequence, the uptake of mineral nutrients (P, K, Fe, Cu, and Zn) is reduced and, in particular, the Na+/K+ ratio becomes very high, interrupting various enzymatic processes and protein synthesis [47]. Plant association with AMF, thanks to their higher soil exploration capacity, showed a total mineral nutrition increase with great K+ accumulation, helping the plants to maintain a lower Na+/K+ ratio and in this way avoid damage to their biological functioning [48];

(3) Intense production of compatible organic solutes: low-molecular-weight and highly soluble compounds, such as proline, glycine, betaine, and soluble sugars, are accumulated to higher levels in AM plants and appear to be positively correlated to fungi plant colonization [49,50]. Production of these solutes can contribute to cellular osmotic adjustment thanks to their key role in detoxifying ROS, protecting membrane integrity, and stabilizing enzymes/proteins [51];

(4) Antioxidant enzyme activity enhancement: several studies have suggested that AM symbiosis intensifies enzyme system activity in ROS detoxification, including that of peroxides, superoxide, hydroxyl radicals, and singlet oxygen and alpha-oxygen, the production of which in plants is strongly influenced by stress factors such as salinity. The effects induced by these compounds on cell metabolism, such as DNA damage, the oxidation of polyunsaturated fatty acids in lipids and of amino acids in proteins, and the deactivation of specific enzymes, appear to be reduced in AM plants, which show generally lower levels of oxidative damage.

Almost all research regarding the role of mycorrhizal symbiosis in enhancing wheat plant tolerance, growth, and yield under salinity stress has been conducted under growth chamber conditions or in a greenhouse, using sterilized soil and salty water or salty soil. At the greenhouse of the National Research Center of Giza (Egypt), Talaat and Shawky [52] studied the key role of AMF in protecting two wheat cultivars (*Triticum aestivum*, cv. Sids 1 and cv. Giza 168) against the detrimental effect of salinity: inoculation with fungi showed their ability to

improve plant productivity; the membrane stability index; photochemical reactions during photosynthesis; the concentrations of N, K+, chlorophyll, carbohydrates, and protein; and the relative water content (RWC).

Abdel-Fattah and Asrar's [53] investigation showed that arbuscular mycorrhizal inoculation (particularly *Funneliformis mosseae*) significantly alleviated the harmful effects of salt stress on wheat plants (*Triticum aestivium*, cv. Henta) grown in saline soil, reducing Na uptake; increasing P, N, K, and Mg contents; and stimulating photosynthetic pigments and some metabolic contents of the wheat plants. Positive results in salt stress conditions were found in pot experiments on *Triticum aestivum* cv. Purna HI-1544, with dual treatment with AM fungi and spermine revealing a great enhancement of wheat yield [54], and a mixed starter culture of native AMF culture (mainly comprising *Rhizophagus intraradices*, *Funneliformis mosseae*, and *Funneliformis geosporum*) resulting in mitigation of the metabolic inhibition of photosynthesis through the maintenance of PSI and PSII integrity and stability [55]. Field experiments regarding the effects of arbuscular mycorrhiza (AM) on wheat plant growth under salinity are very rare, but the beneficial effects under controlled conditions seem to be confirmed. Daei et al. [56] conducted a field experiment in Karaj (Iran) testing and verifying the role of *Glomus etunicatum*, *Glomus mossae*, or *Glomus intraradices* in significantly increasing the growth and nutrient uptake of the mutated Tabasi line (produced by the Institute of Agricultural, Medical and Industrial Research, Tehran, Iran). This experiment was reproduced using the same AM fungi on the same wheat line by Mardukhi et al. [57], confirming enhanced plant capacity in absorbing minerals such as N, Ca, Mg, Fe, Cu, and Mn from salty soil in Alborz province (Iran).

### 3.3.2 Drought stress

Drought represents for plants one of the most common abiotic stresses affecting growth and development [58], resulting in a negative impact on plant survival, development, and productivity [59]. Wilting of the plants and reductions in the net photosynthesis rate, stomatal conductance, water use efficiency, and relative water and total chlorophyll contents are the principal symptoms of drought stress [60]. In addition, the electron transport system is impaired, leading to the formation of activated oxygen [61] and plant stomata closing, resulting in decreased CO2 uptake and an increase of reduction equivalents. As a

consequence, the photosynthetic apparatus is damaged by ROS accumulation, and metabolic processes become involved in the synthesis of secondary plant products (isoprenoids, phenols, or alkaloids) [62]. Many studies on arbuscular mycorrhizal fungi have revealed the importance of their symbiosis with plants in alleviating the negative effects induced by drought [63] and attributed to these microorganisms a key role in the response to this stress [64,65], principally in relation to the following:

(1) Maintenance of water uptake: Mycorrhizal hyphae enter deeper into the soil and explore a great volume in search of water, helping to keep the plant watered [66];
(2) Osmotic adjustment: Some processes like stomatal opening, cellular expansion, and growth are maintained by the mycorrhizal fungus activity, allowing the cells to maintain turgor [67];
(3) Biosynthesis of trehalose: This sugar, consisting of two molecules of glucose and synthesized by the fungus, may contribute to protecting the plant from a lack of water, preserving biological nitrogen fixation and improving the plant's tolerance of drought stress [68,69];

Antioxidant level increase: The concentrations of many antioxidant compounds, such as superoxide dismutase, catalase, and peroxidase, appear to be enhanced in plants colonized by mycorrhizal fungi, resulting in the reduced presence of ROS such as singlet oxygen, superoxides, hydrogen peroxide, and hydroxyl radicals [70]. Expression of drought-related genes in host plants: Mycorrhizal functions may stimulate the expression of some plant genes, including aquaporin genes, the 1-pyrroline-5-carboxylate synthetase (p5cs) gene encoding a rate-limiting enzyme in the biosynthesis of proline, and the 9cisepoxycarotenoid dioxygenase (nced) genes encoding a key enzyme in the biosynthesis of abscisic acid [71]. As a consequence, respectively, the flow of water molecules inside or outside the cells is accelerated [72], one antioxidant action is developed [73], and the stomata are closed to avoid water loss [74]. Inoculation with arbuscular mycorrhizal fungi has been well reported to stimulate wheat growth under drought stress conditions. A metabolomic analysis by Bernardo et al. [75] in a water deficit regime on *Triticum durum* and *Triticum aestivum* wheat cultivars supported the hypothesis of mycorrhizal fungi involvement in enhancing plant response to water stress: inoculation with *Funneliformis mosseae* significantly

improved the plants' biomass production, resulted in a positive trend in water use efficiency (WUE), and reduced oxidative damage, resulting in the root accumulation of compounds known to play an antioxidative role by directly reacting with ROS. Equally, inoculation of Buck Pronto cultivars (*Triticum aestivum*) with *Glomus claroideum* alleviated the deleterious effects of drought stress, revealing significant increases in total dry weight, relative water content (RWC), and leaf chlorophyll content [76]. Field experiments under water-stressed conditions were conducted in Lubbock (Texas) on Steardy (drought-sensitive) and TAM-105 (drought-tolerant) winter wheat cultivars: inoculation with *Funneliformis mosseae* or *Claroideoglomus etunicatum* provided an important enhancement of the yield in both cultivars [77]. *Triticum aestivum* plants, exposed to water stress and grown in soil inoculated with a mixed starter culture of AMF (*Rhizophagus intraradices*, *Funneliformis mosseae*, *F. geosporum*), showed less damage to the structure and function of PSII and PSI systems and exhibited an increase in RWC for both leaf and soil, indicating the ability of AMF hyphae to penetrate deep into the soil and provide moisture to the plants [78]. Finally, beneficial effects of coinoculation with biochar and mycorrhizal fungi on crop N and P supply for dryland wheat production were reported by Solaiman et al. [79].

### 3.3.3 Heavy metals stress

Some metal elements, such as copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), chromium (Cr), molybdenum (Mo), antimony (Sb), and zinc (Zn), are required by plants for numerous enzyme-catalyzed or redox reactions, in electron transfer, and in nucleic acid metabolism [80]. However, high concentrations of them can interfere with essential enzymatic activities by modifying protein structure or by replacing an essential element, resulting in deficiency symptoms such as chlorosis, reduced seed germination [81] and root growth, effects on both photosystems [82], and cell cycle arrest. Nowadays, anthropogenic activities such as industrial waste diffusion and fertilizer application are responsible for heavy metal accumulation in the groundwater and on the soil surface and for subsequent diffusion into plant root systems, representing a real source of stress for these organisms [83].

Nevertheless, arbuscular mycorrhizal fungi seem to have a key role in helping the plants in heavy-metal-contaminated areas. First of all, AM fungal hyphae have a

great ability to explore the soil and, thanks to their surface area for the accumulation of toxic metals, they represent an excellent adsorptive site for the accumulation of cations that prevent entry to the plants [84]. In the extraradical hyphae, heavy metals are frequently precipitated by proteins called glomalines, which are produced by the fungus [85]. Secondly, another positive effect of this symbiosis is localized in the soil where AM fungi release exudates containing citric acid, malic acid, lactic acid, etc. These organic acids form complexes with the metals and reduce their concentration in the soil system [86]. The beneficial effects of AM fungi observed in many recent studies have drawn great interest to a possible role of AM fungi in plant-based strategies for the remediation of highly heavy-metalcontaminated soils. A greenhouse experiment using soil artificially contaminated with high concentrations of zinc, copper, lead, and cadmium showed how mycorrhizal symbiosis between *Funneliformis mosseae* and wheat led to significantly increased root and shoot dry weight, chlorophyll content, and total lipids [87]. Colonization of the same fungus on *Triticum aestivum* cv. Sardari39 plants, under four different Cadmium concentrations (0, 0.3, 0.6, and 0.9 mM Cd), resulted in better growth, higher chlorophyll content, and a higher performance index (PI) for all Cd concentrations compared to the control. Under AM fungus inoculation, the root Cd content was reduced; this might be related to the metal adsorption capability of the relatively large fungal biomass (especially the extraradical hyphal cell walls) associated with the host plant's roots, which may physically minimize or prevent the entry of metals into the host plant [88]. Positive effects of mycorrhiza on wheat (*Triticum durum*) boron (B) uptake under extreme B concentrations were found by Sonmez et al. [89], with the plants' protection from the excessive concentration and uptake of the metal attributed to AM fungus infection. The efficacy of two AM fungi, *Rhizophagus intraradices* and *Claroideoglomus etunicatum*, in the amelioration of As stress in wheat (*Triticum aestivum* L. var. HD-2967) was shown at the Botanical Garden of the Department of Botany (University of Delhi, India), where wheat plants were subjected to four levels of As (0, 25, 50, and 100 mg As kg 1 soil). The formation of AM helped the host plant to overcome Asinduced P deficiency, maintain a favorable P/As ratio, and reduce arsenicinduced oxidative stress (generation of H2O2 and lipid peroxidation), with greater effects under a high As concentration. In addition, colonization with AMF resulted in higher activity levels of antioxidant enzymes (superoxide dismutase, catalase, and guaiacol peroxidase), higher concentrations of antioxidant molecules (carotenoids, proline, and -tocopherol), and increased

activities of both glyoxalase I and glyoxalase II enzymes, revealing the multifarious role of AMF in the alleviation of As toxicity [90]. However, the effect of the prolonged use of agricultural inputs on mycorrhizae naturally present in the soil cannot be excluded: a very interesting experiment on wheat showed how a reduction in agricultural input, after conversion from long-term (more than 20 years) non-mycorrhizal to mycorrhizal crop cultivation, did not show any effects on AMF diversity for the next 3 years [91].

## 3.4 Resistance to pathogens

AM fungi may be used as potential biological control agents for plant diseases [92,93], representing in the agriculture world a valid alternative to chemical pesticides, useful to promoting sustainability and limiting public health hazards. Indeed, AM symbiosis is acknowledged for reducing the damage caused by a wide spectrum of soil-borne pathogens, including fungi and nematodes, which often cause great yield losses [94,95]. In addition, the greater tolerance of mycorrhizal plants against root pathogens could complement innovative multiple-allele quantitative resistance obtained by plant breeding, ensuring high tolerance of plants to pathogens [4]. The mechanisms involved in this biological control seem to be related to the following:

(1) Changes in root growth and morphology: AM colonization induces notable changes in root system morphology, altering the dynamics of pathogens and modifying microbial populations, with the possible stimulation of microbiota components with antagonistic activity toward certain Agronomy 2019, 9, 840 8 of 15 root pathogens [96]. Lucini et al. [97] showed significantly different production of exudates in AMF roots, which can influence the microbiota composition;

(2) Changes in host nutrition: the increased nutrient uptake resulting from AM symbiosis makes the plant more vigorous and, consequently, more resistant, compensating for the loss of root biomass or function caused by pathogens [98];

(3) Competition for colonization sites and photosynthates: both the AM fungi and root pathogens depends on host photosynthates, and they compete for the

carbon compounds reaching the root [99,100]; however, AM fungi have primary access to photosynthates, and the higher carbon demand may inhibit pathogen growth [101];

(4) Activation of defense mechanisms: with AM colonization, the host plant produces a great number of phytoalexins, enzymes of the phenylpropanoid pathway, chitinases, b-1,3-glucanases, peroxidases, pathogenesis-related (PR) proteins, callose, hydroxyproline-rich glycoproteins (HRGP), and phenolics [102] that can act in biological control [103,104].

Many authors have reported that arbuscular mycorrhizal symbiosis can alleviate root disease caused by several pathogens. After inoculation with *Funneliformis mosseae* on spring wheat (*Triticum aestivum* L.), a lower incidence of the root take-all pathogen *Gaeumannomyces graminis* was observed, resulting in an increase in shoot drymass and seed yield, but the best results in reducing pathogen impact were found at 4 weeks with the combined administration of the growthpromoting rhizobacteria (PGPR) *Pseudomonas fluorescens* strain RA56P and the arbuscular mycorrhizal fungus [105]. Equally, *Triticum aestivum* cv. Chinese Spring inoculated with *F. mosseae* revealed a broad-spectrum defense (BSD) response against *Xanthomonas translucens*, where genes and proteins played a regulatory role in the host immune system [106]. The importance of taking into account the inoculum type when considering the use of AMF as biocontrol agents was emphasized by Mustafa et al. [107]. Two commercially available AMF inocula (*Funelliformis mosseae*, Solrize®) and one laboratory inoculum (*Rhizophagus irregularis*) were tested for mycorrhizal protection against *Blumeria graminis* sp. *tritici* in two moderately susceptible and resistant wheat cultivars. The highest protection level against the pathogen was obtained with *F. mosseae* (74%), followed by Solrize (58%) and *R. irregularis* (34%), showing a reduction in the number of conidia with haustorium and an accumulation of polyphenolic compounds at the infection sites. The same positive result was found with the same fungus, *F. mosseae*, when inoculated on *Triticum aestivum* L., confirming both the biocontrol ability of the AMF against *Blumeria graminis* and the mycorrhiza-induced resistance acquired by the plant [108]. Finally, results from Falahian et al. [109] confirmed the enhancing effect of mycorrhizal fungi on Phenylalanine Ammonia Lyase (PAL) activities against *Gaeumannomyces graminis* in wheat plants (*Triticum aestivum*).

## 3.5 Soil aggregation

The soil structure—defined as the particle arrangement of sand, silt, and clay, bound together into aggregates of various sizes—is a crucial aspect of the functioning of the entire ecosystem, because it represents an important source of carbon storage and, at the same time, the exchange site of water, gaseous, and nutritive flows [110]. Nowadays, intensive agricultural practices have adversely influenced the soil structure by decreasing aggregation stability, but it is currently believed that fungal hyphae are one of the main binding agents involved in stabilizing microaggregates [111] for a number of reasons that, in the past and still today, are the research subject of many scientific studies. Firstly, extraradical hyphae ramify around plant roots, exercising a physical penetration force which compresses the soil and results in the reorientation of clay particles [112] and ramification in macroaggregate pores [113]. Secondly, another factor contributing to soil cohesion and strength is connected to the positive effect that mycorrhizal association exerts on plant water status, especially in drought conditions: the greater soil exploration by the fungal hyphae and, consequently, the greater cycles of water from the ground to the plant ensure greater contact between particles and organic matter [114]. Lastly, glomalin, a glycoprotein produced by AM hyphae, is hydrophobic and, thanks to its ability to coat the AM hyphae, has a key role in making the hydrophilic fungal wall able to adhere to hydrophobic surfaces located on soil particles and particulate organic matter [115]. In addition, glomalin production increases C storage and C availability, affecting, respectively, aggregate stability (and, hence, soil structure) [116] and the microbial population [117]. The effect in promoting aggregation is not always the same, and it depends on plant root morphologies and the association type with the fungus; the strongest effect on macroaggregation has been observed with fine roots of the host plant (0.2–1 mm in diameter). The AMF and soil exert on each other a reciprocal influence: on the one hand, as we have just said, these fungi have an effect on the formation of aggregates in the soil; on the other hand, soil quality is fundamental for the very survival of these same beneficial microorganisms. Tillage, for example, destroys the soil structure and, as a consequence, mycorrhizal hyphae. No-tillage practices along with continuous cropping systems (by eliminating fallow periods and/or growing cover crops), using mycorrhizal host crops, and reducing synthetic inputs all enhance the plant–mycorrhizal symbiotic relationship [118]. The capacity of AMF to alleviate the stressful effects of soil compaction on plant growth have been positively verified by many authors [116,119], but few experiments have been

conducted specifically on wheat crops. One of these rare studies is that by Miransari et al. [120] on *Triticum aestivum* cv. Shiraz where, at different levels of soil compaction, inoculation with *F. mosseae* or *C. etunicatum* showed significant increases in the root, shoot ($p = 0.1$), and grain ($p = 0.05$) dry weights.

## 3.6 Conclusions

Over the last few years, the idea of mycorrhizae and their use in the agronomic sector has taken hold in many scientific experiments around the world. Most of the research has focused on the host plant benefits attributed to arbuscular fungi from the points of view of yield and resistance to biotic and abiotic stresses. Great efforts have been made in order to study the processes and metabolic pathways involved in the fungus, aimed at the greater absorption of nutrients and water and greater resistance to pathogens, salinity, and heavy metals. Despite the numerous studies on horticultural and forestry plants, wheat, which is one of the most important food crops worldwide, has been the subject of many tests on mycorrhizal inoculation. With a view to greater environmental sustainability, the selection and cultivation of cereals in agricultural systems with a low environmental impact could be based on the selection of wheat varieties with highly effective mycorrhizal symbiosis. In the last few years, the research has recognized notable differences in plant susceptibility and/or responsiveness to AMF among wheat genotypes that differ in ploidy number or geographic origin [121]. Significant genotypic differences were detected in the ability to form mycorrhizal symbiosis, and some significant markers, representing a Quantitative Trait Locus (QTL), were detected on wheat chromosomes [122]. Future research should therefore not focus only on the AM fungus colonization capacity, but it could take into account the ability of single grain accessions to form a mycorrhiza, based on the results obtained by previous genetic characterization. The identification of molecular markers closely associated with a mycorrhiza could be a very effective tool for selecting highly effective plants for symbiosis and developing wheat varieties suitable for low-environmentalimpact agricultural systems. At the same time, the identification and selection of the most infectious and efficient mycorrhizal fungi in combination with wheat will facilitate their use as biofertilizers to overcome the loss of soil biological fertility, reduce chemical inputs, and alleviate the effects of biotic and abiotic stress.

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# CHAPTER 4

## Genetic variability assessment of 127 *Triticum turgidum* L. accessions for mycorrhizal susceptibility-related traits detection

## 4.1 Introduction

Durum wheat (*Triticum turgidum* L. subsp. *durum* [Desf.] Husn.) is one of the most ancient domesticated grain crops [1] and the only tetraploid wheat subspecies of economic importance. It is widely cultivated around the Mediterranean basin, where it may represent even up to 70% of all wheat acreage, as, for instance, in Algeria and Italy [2]. In such a region, durum wheat is often grown in stressful environmental conditions, with hot and arid environments, where tolerance and resilience to harsh conditions is strongly required. During the *Green Revolution*, breeders imposed a strong selection on durum cultivars based on commercial purposes: local landraces were almost completely replaced by improved semi-dwarf cultivars which showed common characteristics like reduced height and leaf area, limited sprouting and shorter crop cycle [3]. However, such an effort, aimed at improving wheat yield and grain quality, may have resulted in a loss of genetic variability between accessions [4], and decreased resistance to stress [5].

The climate crisis, characterized by rising temperatures and water deficient conditions, and the need for a more sustainable agriculture to mitigate the environmental pollution and meet the increasing demand for food [6], is prompting scientists to recognize the importance of new sources of genetic material [7,8], and of the plant microbe interactions to increase crop production [9,10]. Arbuscular mycorrhizal fungi (AMF) are naturally present in different types of habitats [11], forming associations with the roots of about 80% of all plant species [12]. These associations are mutualistic symbioses where the heterotrophic fungus receives carbon in the form of organic molecules produced by the plant, which in turn obtains mineral nutrients and water absorbed by the fungus [13]. In addition, many studies showed the beneficial role of AMF in improving tolerance and resilience of plants from abiotic and biotic stresses, revealing the great potentiality of these fungi in sustainable and organic agriculture [14,15].

While there is argument as to whether breeding for enhancing plant mycorrhizal interactions [16-18], both mycorrhizal colonization and growth may widely vary among plant accessions, and these traits are considered under genetic control [1921], although a strong environmental effect and a low heritability have been observed [22,23].

Genome-Wide Association studies (GWAS) are these days widely used in plant research to detect Quantitative Trait Loci (QTLs) associated with complex traits such as resistance to biotic stresses [24,25] and yield quality [26,27]. Concerning wheat,

QTL mapping was recently applied to marker-assisted selection (MAS) programs for individual traits contributing to yield enhancement [28,29] and disease resistance [30,31]. Despite the current relevance of the topic, very few studies related to the detection of genetic markers in linkage with chromosome regions involved in AM colonization through GWAS are available [32,19].

In durum wheat, a large variability of mycorrhizal response has been observed among cultivars [33-35], then suggesting that scope exists for optimization of plant interactions with AM fungi. Therefore, a thorough knowledge of the genetic material potentially utilizable for breeding, such as landraces and other naked tetraploid species, is needed to avoid risk of reducing mycorrhizal compatibility of new lines. Here, we analyzed the genetic diversity of 127 accessions belonging to different *T. turgidum* subspecies in relation to their root colonization by two AM fungi (*Funneliformis mosseae* and *Rhizoglomus irregulare*). After a genetic characterization through single-nucleotide polymorphisms (SNPs) markers, a GWAS was implemented to detect Quantitative Trait Nucleotides (QTNs) associated to mycorrhizal susceptibility.

## 4.2 Materials and Methods

### 4.2.1 Plant material and genetic structure analysis

Plant material is part of a tetraploid wheat (*Triticum turgidum*, 2n!=!4x!=!28; AABB genome) collection at was the University of Florence, Italy (Table S1). *T. turgidum* accessions belonging to eight different subspecies (ssp.) were selected among those with the highest genetic diversity in a larger collection: 2 belonging to ssp. *carthlicum*, 28 to ssp. *dicoccon*, 47 to ssp. *durum*, 3 to ssp. *paleocolchicum*, 13 to ssp. *polonicum*, 31 to ssp. *turanicum* and 3 to ssp. *turgidum*. They were genotyped by 35k wheat breeders' Axiom® array using Affymetrix GeneTitan® system at Bristol Genomic Facility (England).

Genomic DNA of 127 accessions was extracted from leaf tissue of each genotype using a standard cetyltrimethylammonium bromide (CTAB) protocol [36] and successively treated with RNase-A (New England Biolabs UK Ltd., Hitchin, UK) according to the manufacturer's instructions. DNA was checked for quality and quantity by electrophoresis on 1% agarose gel and Qubit™ fluorimetric assay (Thermofisher), respectively. The Axiom® Wheat breed Genotyping Arrays 35K was used to genotype the 127 samples for 35.143 SNPs using the Affymetrix

GeneTitan® system at Bristol Genomics Facility according to the procedure described by Affymetrix (Axiom 2.0 Assay Manual Workflow User Guide Rev3).

The array was previously developed for the three genome AABBDD of *Triticum aestivum* and for this reason, working on *T. turgidum,* which lacks D genome, a variant call rate threshold of 92% was used instead of the default value (97%) to account for the lower call rates obtained from tetraploid wheat. Using custom command presented in the Axiom Analysis software, each accession was taken into consideration separately for calculating the number of monomorphic and polymorphic SNP markers, the heterozygosity level and the types of nucleotide substitution. Monomorphic SNP markers and those with missing data points were excluded from analysis. SNP markers were filtered for minimum allele frequency (MAF) greater than 1 % and failure rate lower than 20%, starting from a total of 21051 SNPs.

In order to provide an efficient description of accessions genetic clusters using a few synthetic variables, a discriminant analysis of principal components (DAPC) was implemented, using R software v.3.6. [37] with R\adegenet package [38] and R\poppr package [39]. DAPC first step was the data transformation using principal component analysis (PCA) while the second step was the discriminant analysis performing on the retained principal components (PCs). Groups were identified using k-means, a clustering algorithm which finds a given number (k) of groups maximizing the variation between them. To identify the optimal number of clusters, k-means was run sequentially with increasing values of k, and different clustering solutions were compared using Bayesian Information Criterion (BIC). The optimal clustering solution should represent to the lowest BIC [40].

Subsequently, starting from a matrix of pairwise distances estimated by using Maximum Composite Likelihood (MCL) model, an unrooted, Bayesian tree was obtained by applying the NeighborJoin algorithms [41] in R software with R/ape 3.1 [42]. The branches of the tree corresponding to each genotype were differently colored according to the cluster (found with the DAPC) they belong to. A Bayesian tree and DAPC analysis allowed to investigate levels and patterns of genetic diversity among the examined accessions, utilized to assess the association between the wheat accessions and their root mycorrhizal colonization.

With the purpose of detecting the genetic variation within population and supporting the population structure obtained with DAPC, analysis of molecular

variance (AMOVA) was performed for K=6 subdivision levels using R\poppr package [39].

### 4.2.2 Fungal material

Fungi used in the experiment were: *Funneliformis mosseae* (*F. mosseae*) (T.H. Nicolson & Gerd.) C. Walker & A. Schüßler, isolate IMA1 and *Rhizoglomus irregulare* (*R. irregulare*) (B aszk., Wubet, Renker & Buscot) Sieverd., G.A. Silva & Oehl, isolate IMA6. Isolates were obtained from pot-cultures maintained in the collection of the Microbiology Laboratories of the Department of Agriculture, Food and Environment, University of Pisa, Italy. The fungal inocula were produced in greenhouse on *Trifolium alexandrinum* L. as host plant, grown for 6 months in a mixture (1:1 by volume) of sterilized soil and calcinated clay (OILDRI Chicago, IL, USA). At harvest, roots were cut in ca. 1-cm fragments and mixed with the substrate to form a homogenous crude inoculum mixture, to be used for wheat inoculation. Before starting the experiment, biological activity of such inocula was assessed by using the Mycorrhizal Inoculum Potential (MIP) bioassay, as described in [43].

### 4.2.3 Experimental setup

The experiment examined 127 accessions of *T. turgidum*. Seeds were sown in 6 cell (45 x 55 mm) plug trays filled each with 100 mL of a mixture of *F. mosseae* or *R. irregulare* crude inoculum and sterilized calcined attapulgite clay, 1:1 by volume. Three replicate cells for each tray were utilized for each accession. Plants were grown in an unheated greenhouse and watered with tap water until 10 days after planting (DAP). Then, nutrients were supplied by adding 3 mL of a low P nutrient solution twice a week to each cell, up to the end of the experiment. The nutrient solution contained $NH_4NO_3$ (40 mg/L), $KNO_3$ (101 mg/L), $KH_2PO_4$ (0.5 mg/L), $K_2SO_4$ (14 mg/L), KCl (11 mg/L) from commercial grade compounds.

Throughout the growing period, from April 2019 to early June 2019, daily minimum and maximum outdoor temperatures were in the range of 5.8-17.2 °C and 12.7-25.5 °C, respectively. Every week, trays were randomly moved to avoid position effects.

At harvest, seventy days after emergence, plants were removed, and shoot separated from roots. Shoots were dried in an oven at 50 °C for 72h and weighted, roots were stored for later analysis of mycorrhizal colonization.

### 4.2.4 Mycorrhizal quantification

Plants roots were cleared with 10% KOH in a water bath (80!°C for 15!min) and stained with Trypan blue in lactic acid (0.05%) after 10!min in 2% aqueous HCl. A dissecting microscope (Wild, Leica, Milano, Italy) at 25x!or 40x!magnification was used in order to estimate percentages of AMF colonization by the gridline intersect method [44]. Mycorrhizal colonization on roots samples was also assessed and observed under a Polyvar light microscope (Reichert-Jung, Vienna, Austria) at 125x!and 500x magnification.

### 4.2.5 Phenotypic data analyses

Data of shoot dry weight and root colonization from the wheat accessions were analyzed in SPSS v. 25 using a general linear model including AMF as fixed effect, accession and cluster as random effect and the interaction between AMF and cluster.

Normality and heteroskedasticity were checked and transformations were not required for dry weight. Root colonization data were analyzed after square root transformation. Simple regressions were undertaken on the mean for each accession.

### 4.2.6 Genome-wide association analysis

GWAS was carried out using 127 tetraploid wheat accessions phenotyped for *F. mosseae* and *R. irregulare* fungal colonization. The GWAS was performed using the R\mvp package [45], using the association model of fixed and random model Circulating Probability Unification (FarmCPU) [46]. Principal components calculated on SNPs diversity were included as covariates to capture the population structure existing in the panel. A kinship matrix was calculated on the same SNP set and included as random factor to the model to account to the relatedness among individuals. A multiple test correction was applied with a Bonferroni threshold for a nominal test p value of 0.1. QTNs notation was done

for markers surpassing the Bonferroni threshold while allelic effects and phenotypic variance explained by each marker were derived from the model. Genetic position of QTNs was derived from the CerealsDB database (cerealsdb.uk.net) of Bristol University in order to compare them to QTL already reported in literature. Candidate genes were identified searching gene models within a fixed interval of +/-300 Mb around the corresponding marker position on the Ensembl plant's database (https://plants.ensembl.org). The Pfam 32.0 database was queried to derive the protein domain and the possible function candidate genes.

## 4.3 Results

### 4.3.1 Population structure and genetic diversity of the tetraploid wheat accessions

After SNP dataset filtering, 21,051 SNP markers were identified and used in the statistical analysis for the evaluation of genetic diversity of the 127 tetraploid wheat accessions. The population structure analyzed by the DAPC method identified 6 clusters of genetically related individuals (Fig. 1). The first 100 PCs and three discriminant eigenvalues were retained. The number of detected groups on which DAPC was carried out was established in coincidence with the lowest BIC value using *find.clusters* function (Fig. S1). In this way, the most markedly different alleles among the 6 groups were used as linear discriminants to perform clusters separations, maximizing the variance between groups and minimizing the variance within groups.

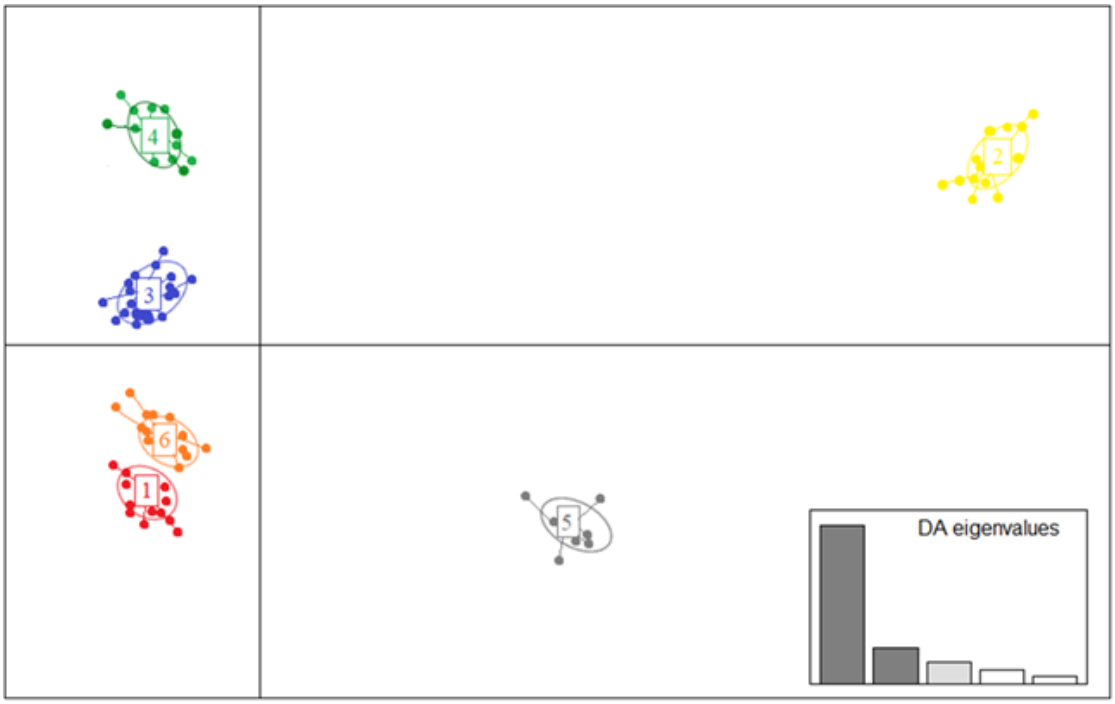


**Fig. 1. Discriminant analysis of principal components (DAPC) for 127 accessions of *Triticum turgidum* L. used for the analysis.** The axes represent the first two Linear Discriminants (LD). Each circle represents a cluster and each dot represents an individual. Numbers represent the different subpopulations identified by DAPC analysis.

The phylogenetic tree, constructed with the Neighbor-Joining algorithm application to a matrix of pairwise distances, better detailed the kinship between accessions and identified clusters that are mainly in agreement with the six subpopulations given by DAPC (Fig. 2). Clusters 1 (red), 2 (yellow), 4 (green), 5 (grey) and 6 (orange) found with DAPC were well identified within the phylogenetic tree. Cluster 3 (blue) was divided into four principal branches which identified four subpopulations, three of them were closer and the fourth more distant.

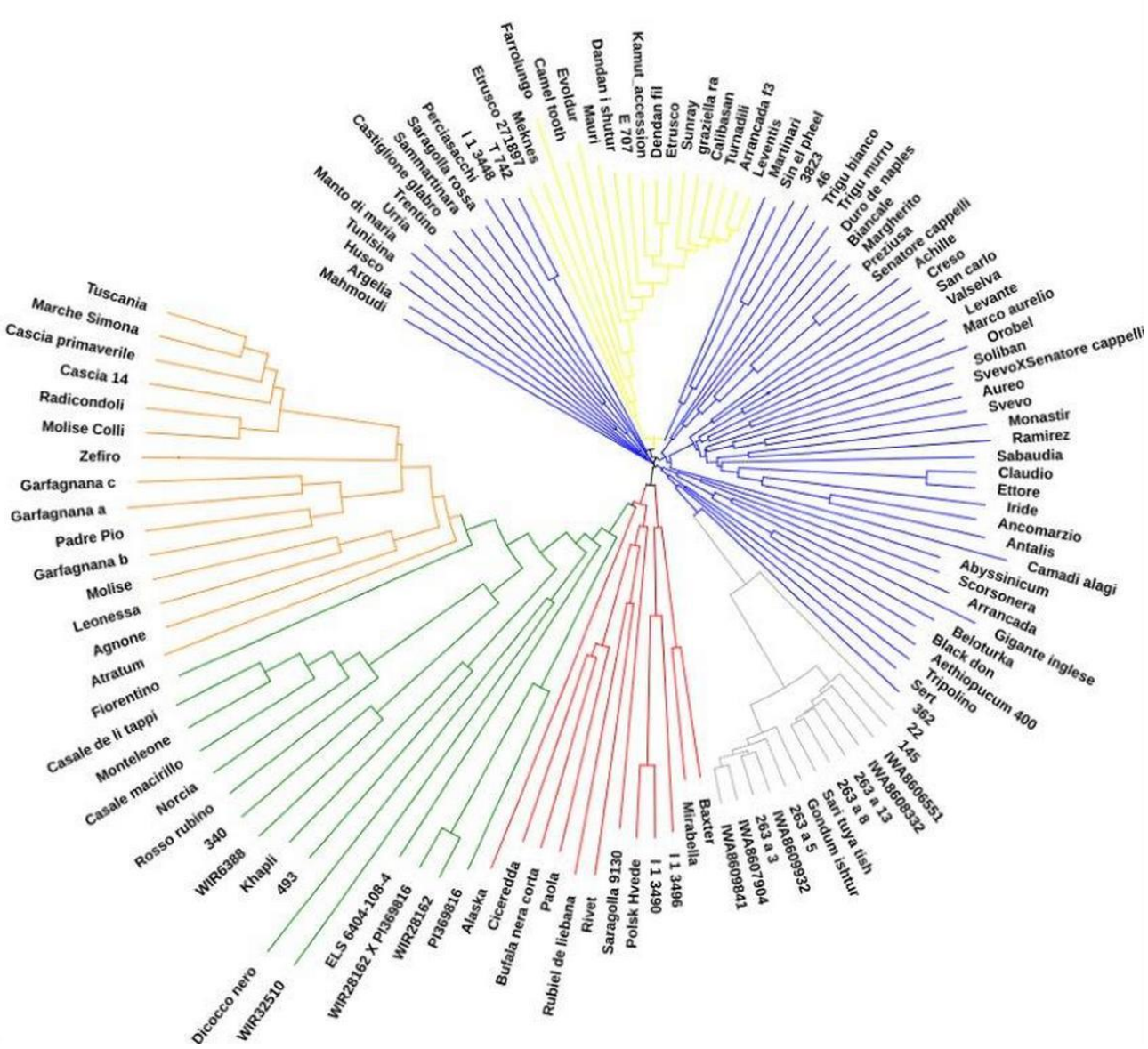


**Fig. 2. Unrooted tree of 127 tetraploid wheat accessions based on single nucleotide polymorphisms (SNPs) markers genetic distance and colored according to discriminant analysis of principal components (DAPC) clusters.** Each tree branches color corresponds to the cluster to which the accession belongs: red for cluster 1, yellow for cluster 2, blue for cluster 3, green for cluster 4, grey for cluster 5 and orange for cluster 6.

Groups identified by DAPC, only partially matched the morphological affiliation of the accessions: however, most of ssp. *durum* accessions (42 out of 47) grouped in cluster 3, most (27) of ssp. *dicoccum* accessions grouped in clusters 6 and 4, and most (26) of ssp. *turanicum* accessions in clusters 2 and 5. Some accessions identified as ssp. *polonicum* (8 out of 13) clustered with the main group

containing ssp. *durum* accessions (cluster 3), while the other (5) accessions were included in cluster 1, which also contained some ssp. *durum* accessions, along with three accessions described as ssp. *turgidum*. Interestingly, the 3 ssp. *palaeocolchicum* accessions and one of ssp. *carthlicum* (PI 341800) grouped in cluster 4, with ssp. *dicoccum* accessions. Another accession described as ssp. *carthlicum* (PI 70738) clustered with ssp. *turanicum* accessions of cluster 5. A single accession of ssp. *dicoccon* (Farrolungo) was included in cluster 2 with different ssp. *turanicum* accessions. AMOVA results for K=6 showed a high variability within accessions (91.67% of the total variance) but confirmed a significant difference between clusters (pvalue= 4.99975e-05) to which 8.33% of the total variance is attributed. In the wake of such results, subsequent analyses were performed following these genetically determined clusters.

### 4.3.2 Mycorrhizal status and colonization level of wheat accessions

The typical symbiotic structures of AMF, such as hyphae, arbuscules, vesicles and spores were observed under the optical microscope, for all accessions (Fig. S2). Roots colonization levels of plants were strongly dependent on the accession, with a high variability both when inoculated with *F. mosseae*, ranging from a minimum of 12.7% (Ramirez, ssp. *durum* of cluster 3) to a maximum of 84.1% (Sin El Pheel, ssp. *polonicum* of cluster 3), and with *R. irregulare*, ranging from 7.0% (Garfagnana b, ssp. *dicoccum* of cluster 6) to 67.7% (Soliban, ssp. *polonicum* of cluster 3) (Tab. S1). ANOVA revealed that a high level of variability occurred within genetic clusters, and that root colonization was affected by the interaction of AM fungus and genetic cluster (Tab. 1). Averaging over each cluster, an effect of genotype was observed, since root colonization by

*F. mosseae* ranged from 47.7% and 50.0% in accessions belonging to clusters 1, 2, 3, and 5, values significantly higher than 33.9% and 25.2% recorded in accessions of cluster 4 and 6, respectively (Tab. 2). A comparable effect of accessions and AMF was observed with *R. irregulare*: clusters 2 and 3 showed the highest colonization level while accessions in cluster 6 showed the lowest level. In addition *R. irregulare* produced a consistently lower level of colonization than with *F. mosseae*: in cluster 6, 5, 1 and 3, root colonization was reduced in average by 33, 27, 25 and 11% compared with *F. mosseae* colonization (Fig. S3). Among the wheat accessions showing the largest colonization differences between *F. mosseae* and *R. irregulare*-inoculated plants, four accessions, all

landraces belonging to the ssp. *dicoccon*, namely Dicocco nero (in cluster 4), and Garfagnana b, Padre pio and Atratum (in cluster 6), were colonized significantly more than 40% by the former AMF. Four accessions, Wir6388 (ssp. *dicoccon*, cluster 4) and Soliban, Ramirez and Abyssinicum (all belonging to ssp. *durum*, cluster 3) were colonized more than 40% by *R. irregulare* (Tab. S1).

The highest root colonization (>60%) by *F. mosseae* was found in accessions included in clusters 3,5 and 1, while the highest (>60%) values of *R.irregulare* colonization were reached in clusters 2,3 and 5. It is of note that some accessions (marked with an asterisk in Tab. S1) –like Marco Aurelio, Antalis, Iride, and Creso- are modern cultivars (released after 1970), which, in average, didn't show a reduction of colonization level in respect of old cultivars and landraces as confirmed by an ANOVA performed on a subset of accessions of ssp. *durum* falling in cluster 3 (P=0.207 and 0.127 respectively for *F. mosseae* and *R. irregulare*, respectively; n: 40).

**Tab. 1.** Nested ANOVA results of the effect of arbuscular mycorrhizal fungal isolates (AMF), genetic cluster of *Triticum turgidum* (Cluster), and wheat accession, on the level of host root mycorrhizal colonization.

| **Source** | **F-value** | **P-value** |
|---|---|---|
| AMF | 18.0 | 0.015 |
| Cluster | 13.2 | <0.001 |
| AMF x Cluster | 3.2 | 0.01 |
| Accession | 6.1 | <0.001 |

**Tab. 2.** Percentage of colonized root length of tetraploid wheats (means and standard errors, SE), by arbuscular mycorrhizal fungus (AMF) and genetic clusters. Means followed by the same letter do not differ significantly (P<0.05). Capital letters (in columns) refer to differences among clusters within *Funneliformis mosseae* or *Rhizoglomus irregulare* treatments. Small letters (in rows) refer to differences between AMF within a cluster. Simple effect test with Sidak correction.

| | AMF | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Funneliformis mosseae* | | | | *Rhizoglomus irregulare* | | | |
| **Cluster** | | | | | | | | |
| | **Mean** | **SE** | | | **Mean** | **SE** | | |
| 1 | 44.0 | 17 | C | b | 33.0 | 14.1 | BC | a |
| 2 | 45.3 | 11.8 | C | a | 43.6 | 13.8 | DE | a |
| 3 | 45.0 | 13.9 | C | b | 40.0 | 14.8 | CDE | a |
| 4 | 33.6 | 10.8 | B | a | 30.2 | 13.8 | B | a |
| 5 | 50.0 | 13.2 | C | b | 36.6 | 12 | BCD | a |
| 6 | 25.7 | 8.3 | A | b | 17.3 | 7.6 | A | a |

**Tab. 3**. Nested ANOVA results of the effect of arbuscular mycorrhizal fungal isolates (AMF), Triticum turgidum clusters and accession, on plant shoot dry weight.

| Effect | F-value | P-value |
|---|---|---|
| **AMF** | 0.02 | 0.889 |
| **Cluster** | 3.79 | 0.023 |
| **AMF x Cluster** | 6.83 | <0.001 |
| **Accession** | 5.69 | <0.001 |

**Tab. 4.** Shoot dry weight of tetraploid wheat plants (means and standard errors, SE), by arbuscular mycorrhizal fungus (AMF) and genetic clusters. Means followed by the same letter do not differ significantly (P<0.05). Capital letters (in columns) refer to differences among clusters within Funneliformis mosseae or Rhizoglomus irregulare treatments. Small letters (in rows) refer to differences between AMF within a cluster. Simple effect test with Sidak correction.

| | **AMF** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | ***Funneliformis mosseae*** | | | | ***Rhizoglomus irregulare*** | | | |
| **Cluster** | | | | | | | | |
| | **Mean** | **SE** | | | **Mean** | **SE** | | |
| **1** | 170.07 | 8.26 | A | a | 167.88 | 9.71 | B | a |
| **2** | 210.97 | 7.18 | BC | a | 233.63 | 8.87 | D | b |
| **3** | 194.99 | 4.00 | B | a | 202.66 | 4.93 | C | a |
| **4** | 219.20 | 10.72 | C | b | 184.27 | 9.60 | BC | a |
| **5** | 204.53 | 7.48 | BC | a | 206.91 | 8.57 | CD | a |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **6** | 155.42 | 6.05 | A | b | 130.49 | 4.22 | A | a |

**Tab. 5.** Significant marker trait associations (MTAs) and corresponding quantitative trait loci (QTLs) for root colonization of tetraploid wheats (Triticum turgidum) by arbuscular mycorrhizal fungi. Chr., chromosome; Physical Pos., position on the chromosome in Mb; R2, variance explained by marker in percentage (%).

| Fungus | QTLs | Marker | Chr. | Physical Pos. | P-value | |
|---|---|---|---|---|---|---|
| | | | | Mb | FarmCPU model | $R^2$ (%) |
| ***R. irregulare*** | QTLamf-2A | AX-94536561 | 2AL | 605.18 | 2.519E-06 | 10.49 |
| | QTLamf-2B | AX-95019471 | 2BL | 693.73 | 3.297E-06 | 13.38 |
| | QTLamf-6A | AX-94438966 | 6AS | 135.24 | 5.424E-06 | 14.96 |
| ***F.mosseae*** | QTLamf-1A | AX-94839893 | 1AS | unknown | 7.182E-07 | 12.91 |

### 4.3.3 Shoot dry weight

The average dry weight of plants from all accessions of *F. mosseae* and *R. irregulare* treatments was 194.1 g and 192.8 g, respectively, but large differences occurred, spanning from 79.7 g in the cross between WIR28162 and PI 369816 (both ssp. *palaeocolchicum*), to 342.2 g in Manto di Maria (ssp. *durum*), after *R. irregulare* inoculation, and from 86.6 g in Polsk Hvede (ssp. *polonicum*) to 331.3 in Dicocco nero (ssp *dicoccon*), after *F. mosseae* inoculation (Tab. S1).

ANOVA (Tab. 3) revealed that a high level of variability on plant shoot dry weight occurred among genetic clusters and among accessions within each cluster. Dry weight was usually lower in accessions belonging to clusters 1 and 6 than in those belonging to cluster 2 and 5, and was affected by AM fungal isolate in accessions belonging to clusters 2, 4 and 6. Actually, inoculation with *F. mosseae* was associated to a higher dry weight of plants in clusters 4 and 6, harboring accessions described as ssp. *dicoccum*, while the opposite trend was observed in accessions belonging to cluster 2 (Tab. 4).

Interestingly, in the remaining clusters, some accessions, such as Saragolla (cluster 1), T-742, Ettore and Iride (cluster 3) and IWA8608332 (cluster 5)

showed a larger dry weight when inoculated with *F. mosseae*. On the contrary, accession 46 (PI 254215) (cluster 3) showed the opposite trend.
At the cluster level, there was a low correlation (Pearson's r <0.5) between plant dry weight and root colonization, with the exception of cluster 2, where a significant ($P < 0.001$), although moderate correlation was detected (Pearson's $r = 0.505$, with *F. mosseae*; $r = 0.518$ with *R. irregulare*).

### 4.3.4 Association mapping and gene identification for AMF colonization

The GWAS analysis identified four unique QTNs for *F. mosseae* and *R. irregulare* (Fig. S4, Fig. S5). One QTN located on chromosome 1A (QTLamf-1A) was explaining 12.9% of the variance of *F. mosseae* colonization. The colonization of *R. irregulare* was associated with three MTAs located on chromosomes 2A (QTLamf-2A), 2B (QTLamf-2B) and 6A (QTLamf-6A), explaining 10,49% to 14,96% of the phenotypic variance (Tab. 5).
172 genes located close to these significant markers were identified and the relevant protein domains were annotated (Tab. S2). The interval to search for candidate genes was set to +- 300 Mb from previous literature [35].

## 4.4 Discussion

### 4.4.1 Population structure and genetic diversity

DAPC analysis based on the 21,051 SNPs provided a global picture of genetic relationship and population structure, also confirmed by AMOVA analysis, of the 127 accessions of different subspecies of tetraploid wheats which grouped in 6 clusters. These clusters only partially matched with the morphological taxonomy of the selected varieties and landraces used in this work, highlighting the occurrence of high genetic variability between accessions belonging to the same subspecies.

*T. turgidum* ssp. *durum* accessions, mainly gathered in cluster 3, were, however, divided into 4 main groups easily identified within the genetic tree. The first two groups, formed by ancestral accessions with different geographical origin (Italy, Tunisia, Ecuador, Hungary and Germany), and old Sicilian durum wheats, respectively, were separated by a cluster of *T. turgidum* spp. *turanicum* accessions with little genetic variability and by a cluster of *T. turgidum* ssp.

*polonicum* accessions. This result is of particular interest since such genetic proximity could be linked to crosses between *durum* and *turanicum* subspecies which originated *T. turgidum* spp. *polonicum* accessions. In addition, within cluster 3, a low genetic variability was showed by modern varieties of *T. turgidum* ssp. *durum* that, unlike the old varieties and landraces, represented a single cluster within the tree. This distribution reflects some results already obtained in previous works which confirmed how the strong selection by breeders -aimed at the constitution of very productive varieties- has considerably reduced the genetic variability between accessions [47,48]. On the contrary, strong genetic differences were highlighted within the second cluster of *T. turgidum* ssp. *turanicum* accessions (cluster 5) characterized by the Iranian origin for the majority of the accessions included. Due to the scarce number of accessions belonging to the ssp. *carthlicum*, *turgidum* and *paleocolchicum*, accurate explanations of analysis results were not possible. Though, the two *T. turgidum* ssp. *paleocolchicum* accessions (WIR28162 and PI_369816) and their cross were located within the same DAPC cluster and on very close branches, at odds with the two *T. turgidum* ssp. *carthlicum* accessions (WIR32510 and 22), whose higher genetic diversity is probably connected to a different origin (Russia and Iraq, respectively). *T. turgidum* ssp. *dicoccon*, assigned to two clusters by the DAPC analysis, identified a clear group within the genetic tree but showed distinctive molecular traits which highlighted a significant genetic diversity already confirmed in previous works [49,50]. Finally, genetic variability was also assessed at accession level: Ciceredda, Paola and Bufala nera corta, defined as the same accession from previous literature [51], were close to each other but constituted different branches within the tree.

### 4.4.2 Phenotypic variability

Our results support the role of the genetic variation of the plant host in determining roots colonization by AMF [19,35], as well as the role of the fungal symbiont. In addition, as previous studies showed [52,53], modern durum wheat varieties were not less colonized by fungi, often presenting, on the contrary, high levels of mycorrhizal susceptibility. This could mean that the breeding processes which led to the establishment of new lines did not impact those chromosomal traits decisive for the association between roots and fungi [35].

However, the use of genetically based clusters identified by DAPC analysis revealed different susceptibility to AMF colonization.

On average, *F. mosseae* showed higher root colonization compared to *R. irregulare*, confirming recent results of Mustafa *et al.* study (2016) [54], where the same fungi were used to inoculate wheat and the highest level of mycorrhizal colonization was reached by *F. mosseae*. Contrary, this observation contradicts De Vita *et al.* research (2018) [35], where wheat roots appeared to be most colonized *R. irregulare*. This inconsistency may be due to the different selection of wheat accessions, though environmental factors such as temperature and light intensity, which have been shown to be involved in determining root colonization [55-58] may have contributed to enhance the mycorrhizal colonization levels.

In addition to light and temperature, other factors related to the host plant, such as root morphology, root to shoot ratio and exudate production, may affect AMF-host interactions [59-61]. In turn, many studies showed how mycorrhizal colonization had a marked impact on these factors, enhancing lateral root formation and branching [62,63], modulating metabolite profiling of root exudates [64], and affecting the root to shoot ratio [65] in the host plant.

Independently on the differences between the two AMF isolates, the high variability in root colonization is confirmed in all wheat subspecies tested, suggesting that such a trait should be considered in breeding programs. Although a low correlation between colonization and plant weight was found in this experiment, it seems possible to breed for maximizing the root colonization, avoiding deleterious effect on plant growth.

### 4.4.3 Quantitative trait loci (QTLs) analysis

Earlier research works identified genetic traits in wheat associated with mycorrhizal colonization. Two studies on *T. aestivum* discovered a positive effect of chromosomes 1A, 5B, 6B, 7B, 5D, and 7D for mycorrhizal response [19] and six QTL regions involved in wheat-AMF associations on chromosomes 3A, 4A and 7A [32]. Recently, seven putative QTLs were linked with durum wheat mycorrhizal susceptibility, and were located on chromosomes 1A, 2B, 5A, 6A, 7A and 7B, were detected [35]. These studies however did not consider the allele pool of wheat subspecies that may contribute with novel variation controlling AMF colonization.

In this study, associations on chromosomes 1A, 2B and 6A were in common with that reported in De Vita *et al.* study (2018) [35] on durum wheat. Nevertheless, QTLs positions identified by this last research were expressed in cM and the approximate conversion to Mb would need further studies to compare their positions with our QTNs. The use of a stringent Bonferroni correction lowered the number of QTN identified because was intended to protect from Type II errors while supporting the relevant of the discussed QTNs. The associations surpassing the threshold co-localized to many traits significantly associated with quantitative phenotypic data –grain yield, biocontrol of *Fusarium*, roots colour- which appear to be affected by AMF-plant interaction [66-68]. Close to QTLamf-6A, QTLs for epistatic effects for flour color traits (QFb.cerz-6AL.2) [69], and for grain length (qgl6A) and weight (qtkw6A) [70] were detected. QTLamf-2A co-localize to QSPS-2A.4 [71] and to the markers DArT3154, DArT3155 and DArT3156, significantly associated to yield-related trait in wheat [72]. The microsatellite Xgwm120 and the SNP 1072874, significantly and respectively associated with QTL for scab [73] and Fusarium head blight (FHB) [69] resistance, were detected in the same chromosome region AX-95019471. Finally, on chromosome 1A, where QTLamf1A for *F. mosseae* colonization was identified, a QTL for grain weight
(QGw1.ccsu-1A) [70] and a QTL for Fusarium head blight resistance (QFhs.nau1AS) [74] were previously observed.

172 functional genes were mapped within +/- 300 Mb interval from the identified QTLs and many of them may related to mycorrhizal colonization by previous works. Genes involved in activities which seemed to be increased during the establishment of AM symbiosis are close to QTLamf-2B, such as the expression of plasma membrane ATPase [75,76], the oxidation-reduction processes [77], the heterochromatin formation in the nucleus [78], the sugars conversion into lipids and their translocation to the extraradical mycelium [79]. Functional genes for hydrolytic enzymes, organic and inorganic N transport, ATP binding protein kinase activities and glycoside transport, associated by recent researches to AMF root colonization [80-85] were located close to QTLamf-2A. Genes related to carbohydrate metabolic process (carbohydrate metabolism and synthesis of cell wall polysaccharide precursors) and Calmodulin binding proteins codification, whose expression resulted higher in AMF colonized plants [72,51] were found close to QTLamf-6A.

## 4.5 Conclusions

This work, which analyzed several accessions of tetraploid wheat belonging to *T. turgidum* ssp. *turgidum*, ssp. *paleocolchicum*, ssp. *carthlicum* and ssp. *polonicum*, in addition to a large collection of other previously not tested *T. turgidum* ssp. *durum* and spp. *dicoccum*, , made it possible to identify four QTN possibly contributing to mycorrhizal susceptibility. These results could enrich future breeding activities aimed at developing new grains on the basis of genetic diversity on low or high susceptibility to mycorrhization, and, possibly, maximizing the symbiotic effects.

## 4.6 Supplementary materials

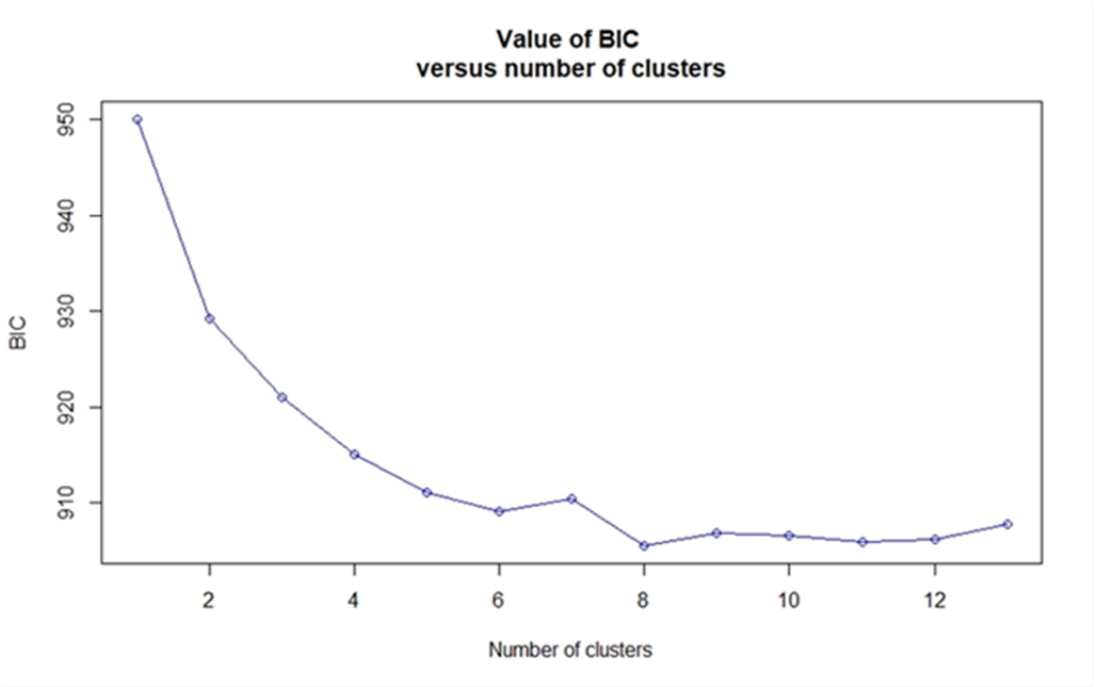


**Fig. S1. Statistical determination of the optimum number of clusters by discriminant analysis of principal components (DAPC).** The elbow in the curve matches the smallest BIC, and clearly indicates 6 clusters should be retained.

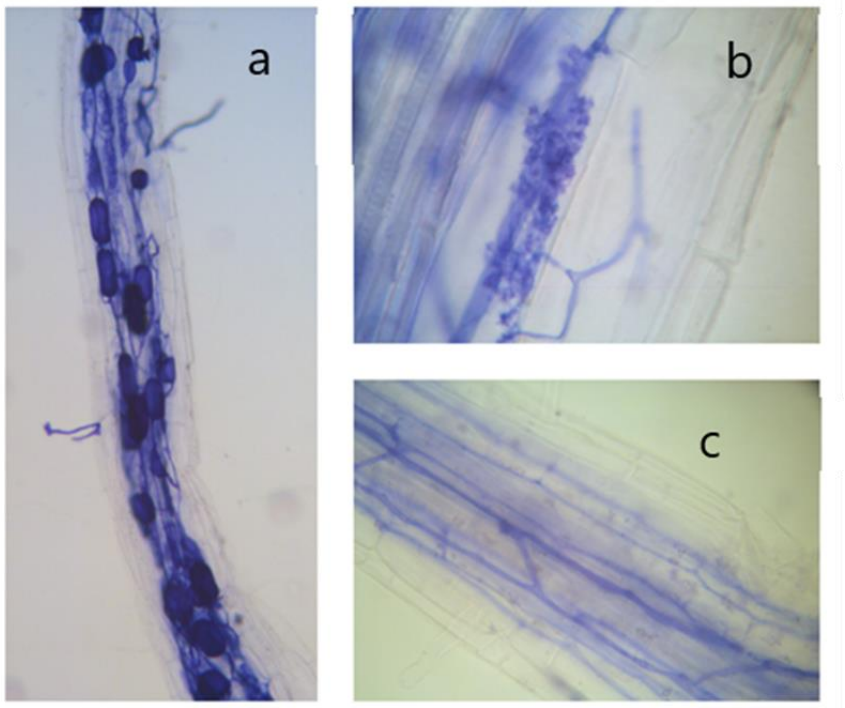


**Fig. S2. Arbuscular mycorrhizal colonization of *Funneliformis mosseae* (FM) in Aureo (*Triticum turgidum* L.) root.** (a) Intraradical vesicles and intraradical spores. (b) Arbuscular coil. (c) hyphal coils.

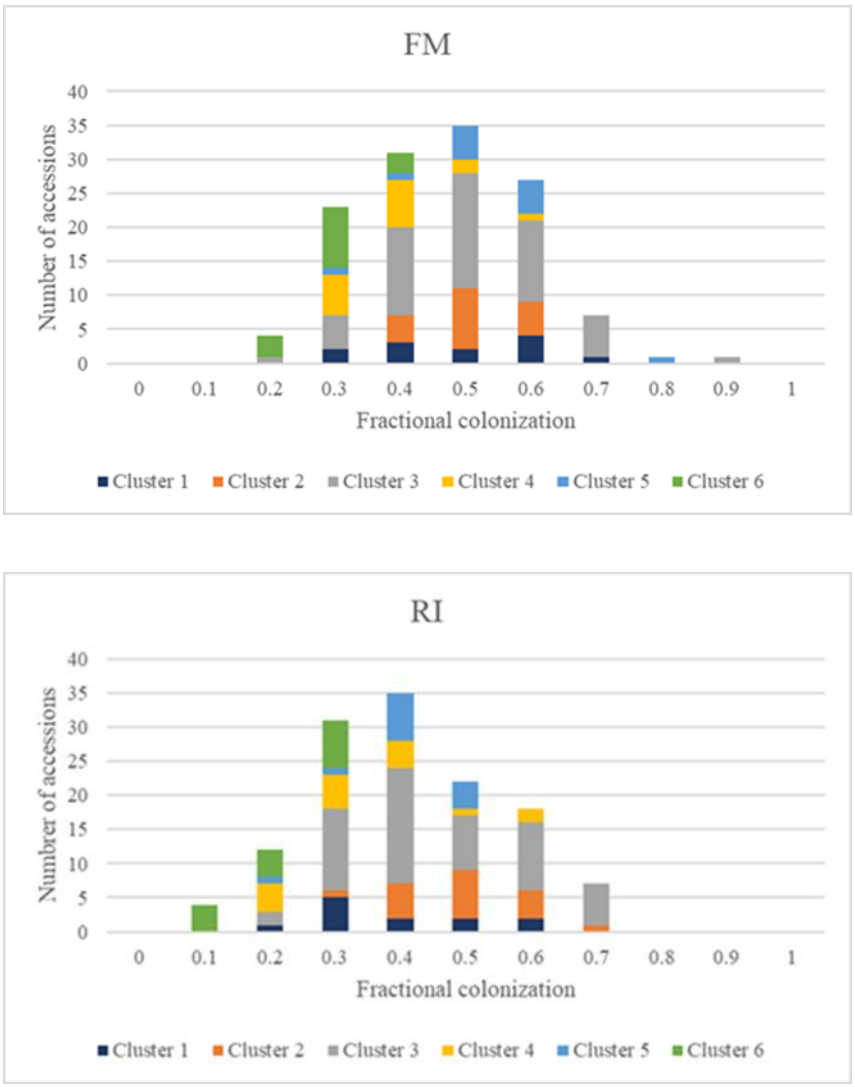


**Fig. S3. Frequency distribution of means for *Funneliformis mosseae* (FM) or *Rhizoglomus irregulare* (RI) in 127 tetraploid wheat accessions at 70 days after emergence.**

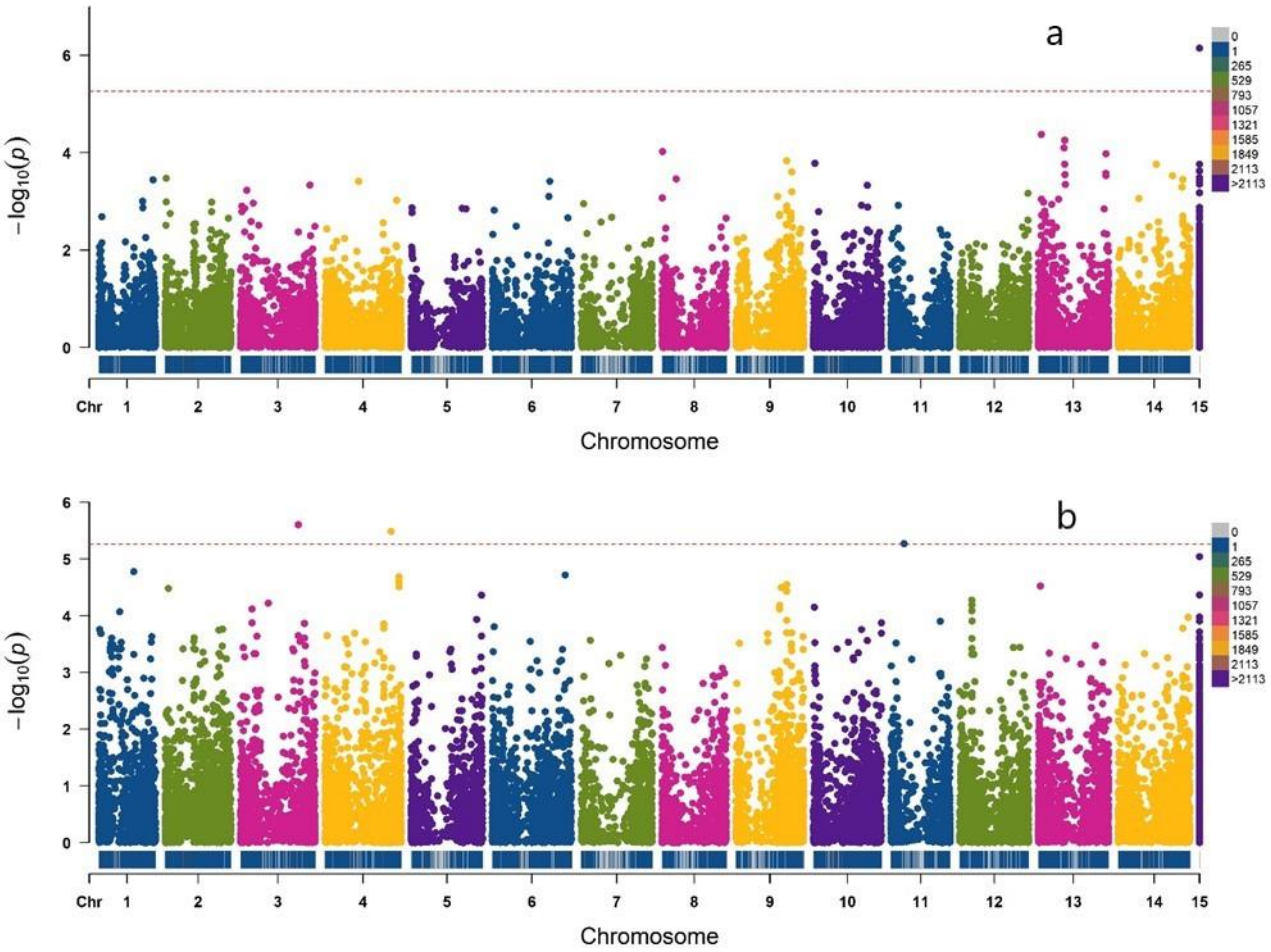


**Figure S4. Manhattan plot ( log10[P]) genome-wide association plot) of a genomewide association study on 130 tetraploid wheat accessions colonized by *Funneliformis mosseae* (a) and *Rhizoglomus irregulare* (b).** The genome-wide significance level is set at
5.462E-06 and plotted as the dotted line.

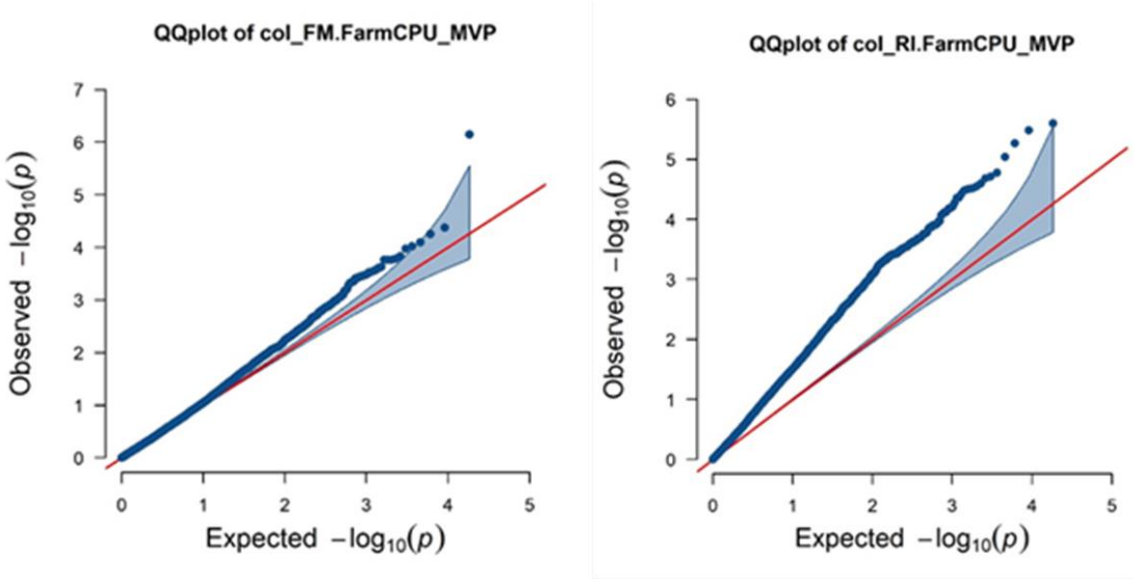


**Figure S5. Quantile-quantile (QQ) plot. Comparison of primary GWAS P-values to those expected for a null distribution.**

**Table S1. List of accessions of *Triticum turgidum* subspecies included in the experiment and phenotypic data related to AMF colonization and dry weight.**

# ACCESSION LIST

| Accessions | Identifier number | Triticum turgidum ssp. | origin | cluster DAPC | *F.mosseae* % roots colonization | | *R.irregulare* % roots colonization | | *F.mosseae* dry weight mg plant-1 | | *R.irregulare* dry weight mg plant-1 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Mean | Std. Error of Mean | Mean | Std. Error of Mean | Mean | Std. Error of Mean | Mean | Std. Error of Mean |
| Paola | | durum | Italy | 1 | 67,50 | 6,58 | 41,20 | 3,06 | 154,45 | 13,86 | 120,48 | 15,17 |
| Manto_di_maria | | durum | Italy | 3 | 40,80 | 6,32 | 44,25 | 3,95 | 244,83 | 19,10 | 342,22 | 45,72 |
| Ramirez* | | durum | France | 3 | 12,70 | 2,13 | 23,75 | 0,95 | 127,10 | 6,42 | 181,71 | 70,21 |
| Ettore* | | durum | Italy | 3 | 28,37 | 4,58 | 37,33 | 2,88 | 167,90 | 4,68 | 143,60 | 10,10 |
| Claudio* | | durum | Italy | 3 | 41,83 | 7,90 | 29,63 | 4,37 | 100,42 | 9,33 | 114,83 | 3,94 |
| Ancomarzio* | | durum | Italy | 3 | 49,23 | 12,63 | 55,40 | 4,18 | 186,36 | 17,64 | 211,24 | 30,67 |
| Svevo* | | durum | Italy | 3 | 33,43 | 8,47 | 32,20 | 4,22 | 155,50 | 4,09 | 150,16 | 38,39 |
| Achille* | | durum | Italy | 3 | 58,97 | 3,49 | 51,93 | 7,42 | 137,11 | 27,67 | 157,17 | 8,69 |
| Preziusa | | durum | Italy | 3 | 34,70 | 2,30 | 28,20 | 7,36 | 195,75 | 10,25 | 126,08 | 22,34 |
| Marco_aurelio* | | durum | Italy | 3 | 64,57 | 5,98 | 47,93 | 5,73 | 187,05 | 31,84 | 232,81 | 13,87 |
| Biancale | | durum | Italy | 3 | 31,43 | 4,45 | 34,43 | 1,68 | 216,17 | 19,48 | 204,50 | 28,00 |
| Duro_de_naples | PI192525 | durum | Italy | 3 | 31,70 | 4,80 | 23,53 | 3,51 | 153,67 | 20,46 | 157,33 | 11,66 |
| Margherito | | durum | Italy | 3 | 29,80 | 4,29 | 33,13 | 3,62 | 178,50 | 9,99 | 183,17 | 16,64 |
| Trigu_murru | | durum | Italy | 3 | 44,23 | 3,60 | 30,20 | 4,09 | 195,67 | 19,66 | 231,50 | 22,50 |
| Abyssinicum | PI352445 | durum | Ethiopia | 3 | 30,23 | 3,49 | 44,70 | 3,61 | 170,90 | 14,20 | 230,50 | 47,63 |
| Beloturka | CItr5014 | durum | Russian | 3 | 38,07 | 6,71 | 50,80 | 5,98 | 192,16 | 43,06 | 256,31 | 19,34 |
| Black_don | PI5645 | durum | Russian | 3 | 37,03 | 3,91 | 50,93 | 5,69 | 222,50 | 12,82 | 228,17 | 24,42 |
| Tripolino | PI157959 | durum | Italy | 3 | 65,33 | 0,92 | 41,33 | 5,02 | 145,00 | 6,66 | 235,33 | 16,59 |
| Sammartinara | PI157958 | durum | Italy | 3 | 36,03 | 4,33 | 18,47 | 0,77 | 194,67 | 12,82 | 200,96 | 18,82 |
| Castiglione_glabro | PI157974 | durum | Italy | 3 | 50,97 | 4,32 | 37,67 | 2,90 | 172,50 | 10,05 | 167,62 | 6,83 |
| Mahmoudi | CItr3809 | durum | Tunisia | 3 | 41,03 | 6,81 | 30,47 | 5,98 | 207,50 | 19,86 | 210,33 | 19,20 |
| Tunisina | PI157967 | durum | Italy | 3 | 44,50 | 4,39 | 24,73 | 2,72 | 205,83 | 18,25 | 228,96 | 25,14 |
| Valselva* | PI367212 | durum | Italy | 3 | 52,83 | 5,76 | 31,30 | 6,49 | 162,11 | 41,11 | 140,98 | 24,45 |
| Levante* | | durum | Italy | 3 | 50,43 | 1,68 | 37,67 | 0,37 | 218,83 | 11,09 | 231,33 | 15,39 |
| Camadi_alagi | PI192130 | durum | Ethiopia | 3 | 35,40 | 2,19 | 26,67 | 2,34 | 225,83 | 13,95 | 252,33 | 9,39 |
| Trentino | PI157965 | durum | Italy | 3 | 45,53 | 9,75 | 39,87 | 3,33 | 210,17 | 10,40 | 220,00 | 19,47 |
| Antalis* | | durum | France | 3 | 61,80 | 1,30 | 39,13 | 5,15 | 174,07 | 29,28 | 164,83 | 19,66 |
| Iride* | | durum | Italy | 3 | 65,60 | 0,59 | 63,73 | 1,24 | 249,83 | 4,69 | 205,04 | 14,87 |
| Creso* | | durum | Italy | 3 | 67,13 | 1,53 | 63,40 | 1,15 | 220,83 | 16,60 | 233,38 | 6,55 |
| Orobel* | | durum | France | 3 | 50,50 | 3,90 | 53,83 | 1,45 | 245,00 | 26,75 | 194,33 | 26,93 |
| San_carlo* | | durum | Italy | 3 | 47,00 | 2,75 | 41,50 | 3,89 | 242,67 | 31,81 | 181,33 | 34,90 |
| Aethiopucum_400 | PI352446 | durum | Ethiopia | 3 | 37,70 | 5,91 | 29,37 | 3,96 | 198,08 | 12,34 | 167,90 | 15,68 |
| Scorsonera | PI264955 | durum | Italy | 3 | 48,40 | 12,26 | 24,97 | 4,48 | 201,80 | 14,03 | 278,67 | 41,46 |
| Trigu_bianco | | durum | Italy | 3 | 50,83 | 2,04 | 53,85 | 2,55 | 244,20 | 4,54 | 314,75 | 22,75 |
| Saragolla_rossa | PI231380 | durum | Italy | 3 | 42,00 | 1,75 | 51,40 | 2,47 | 252,17 | 17,27 | 270,71 | 45,52 |
| Urria | PI157968 | durum | Italy | 3 | 45,03 | 3,47 | 32,70 | 5,95 | 242,17 | 16,22 | 251,00 | 46,35 |
| Monastir* | | durum | France | 3 | 37,67 | 3,80 | 23,40 | 0,81 | 139,97 | 28,18 | 216,10 | 8,40 |
| Aureo* | | durum | Italy | 3 | 52,03 | 2,99 | 56,10 | 4,78 | 234,00 | 14,70 | 247,83 | 9,58 |

| Accessions | Identifier number | Triticum turgidum ssp. | origin | cluster DAPC | *F.mosseae* % roots colonization | | *R.irregulare* % roots colonization | | *F.mosseae* dry weight mg plant-1 | | *R.irregulare* dry weight mg plant-1 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Mean | Std. Error of Mean | Mean | Std. Error of Mean | Mean | Std. Error of Mean | Mean | Std. Error of Mean |
| Senatore_cappelli | PI352414 | durum | Italy | 3 | 35,13 | 2,98 | 28,00 | 1,93 | 189,46 | 33,47 | 187,00 | 16,30 |
| Bufala_nera_corta | PI157985 | durum | Italy | 1 | 26,57 | 5,54 | 17,53 | 2,35 | 142,59 | 26,38 | 170,85 | 17,14 |
| Ciceredda | | durum | Italy | 1 | 33,83 | 10,73 | 26,90 | 5,11 | 141,75 | 4,63 | 144,36 | 9,67 |
| Sabaudia | PI352418 | durum | Italy | 3 | 62,40 | 1,55 | 63,63 | 10,50 | 248,74 | 28,76 | 234,18 | 38,24 |
| Saragolla_9130 | PI9130 | turanicum | Italy | 1 | 43,10 | 8,27 | 23,57 | 10,08 | 204,45 | 22,43 | 115,90 | 9,56 |
| Atratum | PI221398 | dicoccon | Serbia | 6 | 36,53 | 1,97 | 9,07 | 0,99 | 123,37 | 10,55 | 132,10 | 3,49 |
| Polsk_Hvede | PI361757 | polonicum | Denmark | 1 | 35,10 | 4,00 | 23,05 | 7,15 | 86,63 | 0,87 | 101,32 | 8,19 |
| Rivet | PI278221 | turgidum | United Kingdom | 1 | 59,90 | 3,56 | 45,05 | 5,25 | 147,08 | 18,90 | 136,10 | 2,60 |
| Rubiel_de_liebana | PI191203 | turgidum | Spain | 1 | 59,65 | 7,05 | 34,03 | 7,31 | 163,82 | 67,34 | 142,68 | 6,55 |
| I_1_3490 | PI272570 | polonicum | Hungary | 1 | 52,90 | 1,80 | 51,00 | 8,60 | 175,81 | 11,19 | 187,47 | 34,97 |
| Baxter | PI608017 | polonicum | United States | 1 | 23,27 | 2,78 | 29,60 | 2,33 | 164,83 | 18,64 | 188,25 | 7,25 |
| Mirabella | PI352488 | polonicum | Italy | 1 | 36,50 | 12,28 | 23,53 | 7,30 | 188,40 | 21,58 | 191,00 | 18,25 |
| I_1_3496 | PI272564 | polonicum | Hungary | 1 | 36,20 | 7,10 | 31,03 | 4,88 | 242,50 | 20,00 | 227,49 | 29,03 |
| Alaska | CItr5988 | turgidum | United States | 1 | 53,50 | 1,87 | 55,87 | 1,78 | 222,83 | 15,34 | 269,16 | 29,68 |
| graziella_ra | | turanicum | Italy | 2 | 38,87 | 6,85 | 30,23 | 3,21 | 154,40 | 7,54 | 169,15 | 8,02 |
| Dandan-i-shutur | PI127106 | turanicum | Afghanistan | 2 | 47,70 | 2,55 | 39,07 | 15,84 | 176,00 | 28,33 | 170,41 | 22,93 |
| Farrolungo | PI278350 | dicoccon | Italy | 2 | 43,65 | 5,55 | 46,00 | 5,65 | 176,02 | 31,02 | 171,31 | 20,31 |
| Kamut | | turanicum | North Africa | 2 | 39,70 | 5,76 | 31,40 | 2,14 | 171,50 | 14,60 | 202,04 | 40,22 |
| Meknes | PI192658 | turanicum | Morocco | 2 | 36,20 | 9,39 | 48,87 | 2,71 | 191,73 | 45,60 | 203,17 | 45,33 |
| Arrancada_f3 | PI184543 | turanicum | Portugal | 2 | 51,97 | 0,87 | 47,90 | 5,00 | 208,33 | 41,70 | 213,40 | 28,63 |
| Mauri | PI347132 | turanicum | Afghanistan | 2 | 55,13 | 1,95 | 50,47 | 5,55 | 202,86 | 23,90 | 223,73 | 14,48 |
| Sunray | CItr11390 | turanicum | United States | 2 | 40,27 | 8,45 | 37,53 | 13,26 | 205,67 | 6,93 | 228,41 | 14,51 |
| Etrusco_271897 | PI271897 | durum | Italy | 2 | 38,30 | 7,90 | 28,30 | 3,50 | 262,50 | 26,39 | 233,00 | 27,76 |
| Etrusco_1 | | turanicum | Italy | 2 | 55,30 | 3,70 | 33,10 | 3,84 | 269,00 | 25,00 | 237,45 | 48,38 |
| Tumadili | PI166959 | turanicum | Turkey | 2 | 40,87 | 13,71 | 41,97 | 3,44 | 184,31 | 30,92 | 246,47 | 9,47 |
| Evoldur | | durum | Italy | 2 | 58,83 | 7,84 | 43,63 | 0,65 | 257,73 | 34,21 | 257,62 | 36,09 |
| E_707 | PI283795 | turanicum | Afghanistan | 2 | 42,00 | 4,50 | 61,53 | 8,14 | 212,67 | 28,09 | 258,27 | 26,64 |
| Camel__tooth | PI321743 | turanicum | Afghanistan | 2 | 40,47 | 2,22 | 40,07 | 4,49 | 258,17 | 13,22 | 272,44 | 48,35 |
| Dendan_fil | PI337643 | turanicum | Afghanistan | 2 | 49,83 | 7,77 | 52,50 | 5,05 | 246,21 | 14,81 | 283,47 | 47,60 |
| Calibasan | PI166308 | turanicum | Turkey | 2 | 46,20 | 8,38 | 50,47 | 4,59 | 207,64 | 21,94 | 290,66 | 5,70 |
| Leventis | PI306665 | turanicum | France | 2 | 44,80 | 3,20 | 58,27 | 11,32 | 209,47 | 20,36 | 324,49 | 39,99 |
| 3823 | PI190951 | polonicum | Portugal | 3 | 57,93 | 1,44 | 35,15 | 15,15 | 131,00 | 27,11 | 119,82 | 42,32 |
| 362 | PI68287 | turanicum | Azerbaijan | 3 | 50,30 | 0,91 | 49,43 | 2,64 | 143,98 | 6,81 | 131,20 | 28,19 |
| Sert | PI167481 | turanicum | Turkey | 3 | 27,77 | 3,13 | 18,27 | 5,78 | 144,55 | 6,06 | 132,75 | 21,77 |
| T_742 | PI352487 | polonicum | Germany | 3 | 43,13 | 3,07 | 36,53 | 9,94 | 192,51 | 9,11 | 138,59 | 13,86 |
| SvevoXSenatore_cappelli | | durum | Italy | 3 | 53,43 | 6,83 | 32,17 | 2,18 | 220,41 | 40,30 | 156,50 | 9,83 |
| Husco | PI286547 | polonicum | Ecuador | 3 | 29,37 | 4,16 | 22,87 | 2,98 | 216,17 | 33,06 | 166,60 | 17,58 |

| Accessions | Identifier number | Triticum turgidum ssp. | origin | cluster DAPC | *F.mosseae* % roots colonization | | *R.irregulare* % roots colonization | | *F.mosseae* dry weight mg plant-1 | | *R.irregulare* dry weight mg plant-1 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Mean | Std. Error of Mean | Mean | Std. Error of Mean | Mean | Std. Error of Mean | Mean | Std. Error of Mean |
| I-1-3448 | PI272590 | polonicum | Hungary | 3 | 49,20 | 2,70 | 41,67 | 3,62 | 193,82 | 17,47 | 179,99 | 26,22 |
| Gigante_inglese_b | PI184526 | turanicum | Portugal | 3 | 39,27 | 4,05 | 43,20 | 3,67 | 190,77 | 21,28 | 191,27 | 15,84 |
| Perciasacchi | | turanicum | Italy | 3 | 43,65 | 2,25 | 27,17 | 3,35 | 211,67 | 29,56 | 191,33 | 38,53 |
| Arrancada | PI191823 | polonicum | Portugal | 3 | 53,75 | 0,65 | 63,20 | 1,40 | 128,50 | 27,50 | 191,50 | 43,50 |
| Soliban | | durum | Italy | 3 | 41,23 | 1,85 | 67,73 | 6,14 | 178,67 | 21,68 | 213,33 | 33,59 |
| 46 | PI254215 | polonicum | Iraq | 3 | 57,17 | 4,04 | 51,97 | 4,99 | 138,43 | 12,72 | 214,67 | 24,92 |
| Sin_el_pheel | PI208911 | polonicum | Iraq | 3 | 84,05 | 7,25 | 62,90 | 18,00 | 233,83 | 54,94 | 241,87 | 18,76 |
| Argelia | | durum | Italy | 3 | 44,77 | 2,08 | 34,40 | 8,00 | 261,52 | 26,13 | 253,00 | 26,50 |
| Martinari | PI134945 | polonicum | Portugal | 3 | 40,20 | 1,81 | 54,87 | 1,83 | 277,50 | 23,82 | 324,48 | 33,28 |
| WIR28162XPI_369816 | | paleocolchicum | | 4 | 28,40 | 4,70 | 18,93 | 1,92 | 120,80 | 9,18 | 79,69 | 11,07 |
| WIR28162 | PI349050 | paleocolchicum | Georgia | 4 | 49,75 | 4,75 | 21,73 | 3,43 | 110,75 | 9,75 | 94,40 | 10,71 |
| PI_369816 | PI369816 | paleocolchicum | Russian | 4 | 35,17 | 1,17 | 18,70 | 3,10 | 119,10 | 11,98 | 103,34 | 19,32 |
| ELS 6404-108-4 | CItr14750 | dicoccon | Ethiopia | 4 | 24,33 | 3,79 | 32,97 | 4,65 | 139,83 | 18,81 | 122,08 | 10,00 |
| Dicocco_nero | | dicoccon | Italy | 4 | 36,40 | 4,46 | 14,00 | 0,76 | 331,30 | 6,56 | 187,50 | 30,58 |
| Fiorentino | | dicoccon | Italy | 4 | 22,97 | 2,29 | 22,17 | 3,58 | 202,30 | 15,83 | 191,50 | 16,75 |
| Casale_de_li_tappi | | dicoccon | Italy | 4 | 33,03 | 1,04 | 25,57 | 1,61 | 221,83 | 17,55 | 191,95 | 15,35 |
| Casale_macirillo | | dicoccon | Italy | 4 | 21,60 | 1,45 | 36,30 | 7,58 | 203,16 | 38,81 | 193,12 | 19,04 |
| 493 | CItr7962 | dicoccon | Ethiopia | 4 | 52,87 | 2,56 | 54,40 | 2,26 | 224,17 | 14,13 | 193,80 | 24,88 |
| Rosso_rubino | | dicoccon | Italy | 4 | 28,20 | 1,42 | 19,53 | 0,69 | 206,09 | 11,93 | 196,37 | 12,01 |
| 340 | CItr7779 | dicoccon | Ethiopia | 4 | 33,83 | 3,78 | 23,03 | 0,69 | 293,38 | 25,28 | 201,67 | 35,33 |
| Norcia | | dicoccon | Italy | 4 | 30,20 | 1,12 | 22,87 | 2,11 | 232,20 | 32,71 | 208,42 | 29,26 |
| Khapli | CItr4013 | dicoccon | India | 4 | 39,53 | 10,97 | 45,67 | 2,79 | 253,41 | 37,04 | 214,33 | 14,44 |
| WIR32510 | PI341800 | carthlicum | Russian | 4 | 47,73 | 2,44 | 39,10 | 5,16 | 251,17 | 17,90 | 214,83 | 18,49 |
| Monteleone | | dicoccon | Italy | 4 | 23,10 | 0,30 | 30,33 | 1,37 | 295,20 | 38,39 | 251,73 | 29,74 |
| WIR6388 | PI349043 | dicoccon | Georgia | 4 | 31,60 | 3,85 | 57,73 | 4,76 | 291,73 | 25,97 | 303,54 | 46,32 |
| IWA8608332 | PI624429 | turanicum | Iran | 5 | 44,77 | 3,26 | 34,70 | 9,20 | 206,77 | 7,59 | 144,50 | 15,00 |
| IWA8609932 | PI625164 | turanicum | Iran | 5 | 75,37 | 1,66 | 43,63 | 10,03 | 206,81 | 21,57 | 169,86 | 23,50 |
| 263_a_5 | PI254198 | turanicum | Iran | 5 | 48,30 | 3,57 | 28,70 | 3,02 | 195,21 | 29,39 | 172,09 | 11,61 |
| 263_a_8 | PI254201 | turanicum | Iran | 5 | 43,03 | 2,73 | 39,43 | 12,32 | 196,43 | 11,29 | 175,17 | 19,13 |
| 22 | PI70738 | carthlicum | Iraq | 5 | 28,80 | 1,10 | 17,30 | 2,46 | 192,25 | 19,25 | 197,59 | 19,76 |
| IWA8607904 | PI624217 | turanicum | Iran | 5 | 55,33 | 2,51 | 37,05 | 0,95 | 190,00 | 9,41 | 199,00 | 27,50 |
| 263_a_13 | PI254206 | turanicum | Iran | 5 | 37,93 | 4,48 | 38,40 | 9,72 | 173,08 | 23,77 | 213,87 | 34,69 |
| IWA8609841 | PI625155 | turanicum | Iran | 5 | 44,43 | 7,38 | 41,25 | 0,95 | 176,00 | 25,90 | 216,50 | 19,50 |
| 263_a_3 | PI254196 | turanicum | Iran | 5 | 45,63 | 1,34 | 38,80 | 7,10 | 176,83 | 23,71 | 235,51 | 73,37 |
| Gondum_ishtur | PI317492 | turanicum | Afghanistan | 5 | 53,53 | 12,57 | 37,47 | 4,70 | 227,34 | 42,92 | 237,84 | 8,63 |
| IWA8606551 | PI623656 | turanicum | Azerbaijan | 5 | 58,17 | 5,62 | 34,10 | 1,00 | 251,70 | 42,29 | 240,00 | 8,50 |
| 145 | PI225331 | turanicum | Iran | 5 | 58,65 | 7,75 | 46,57 | 4,86 | 220,03 | 26,53 | 244,06 | 36,41 |

| Accessions | Identifier number | Triticum turgidum ssp. | origin | cluster DAPC | *F.mosseae* % roots colonization | | *R.irregulare* % roots colonization | | *F.mosseae* dry weight mg plant-1 | | *R.irregulare* dry weight mg plant-1 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Mean | Std. Error of Mean | Mean | Std. Error of Mean | Mean | Std. Error of Mean | Mean | Std. Error of Mean |
| Sari_tuya_tish | PI290530 | turanicum | Hungary | 5 | 52,25 | 3,05 | 40,80 | 0,90 | 269,00 | 5,00 | 262,75 | 40,25 |
| Garfagnana_b | | dicoccon | Italy | 6 | 20,03 | 3,85 | 7,00 | 1,46 | 121,67 | 6,50 | 99,89 | 1,80 |
| Garfagnana_a | | dicoccon | Italy | 6 | 18,35 | 4,55 | 18,00 | 3,56 | 117,00 | 7,20 | 103,09 | 4,95 |
| Marche_simona | | dicoccon | Italy | 6 | 24,13 | 4,07 | 21,97 | 4,07 | 145,01 | 10,36 | 105,17 | 7,12 |
| Cascia_primaverile | | dicoccon | Italy | 6 | 31,53 | 2,55 | 21,20 | 1,96 | 116,92 | 16,21 | 114,53 | 3,31 |
| Padre_pio | | dicoccon | Italy | 6 | 28,57 | 6,12 | 9,77 | 1,59 | 175,89 | 13,49 | 116,16 | 7,03 |
| Molise | | dicoccon | Italy | 6 | 25,17 | 3,88 | 21,97 | 0,67 | 112,27 | 6,85 | 118,93 | 3,28 |
| Tuscania | | dicoccon | Italy | 6 | 23,93 | 1,50 | 24,53 | 2,28 | 122,33 | 14,57 | 123,13 | 8,81 |
| Agnone | | dicoccon | Italy | 6 | 24,23 | 4,55 | 24,67 | 1,23 | 178,77 | 6,95 | 124,37 | 6,95 |
| Garfagnana_c | | dicoccon | Italy | 6 | 15,17 | 2,83 | 13,57 | 2,34 | 180,39 | 8,44 | 134,50 | 18,85 |
| Leonessa | | dicoccon | Italy | 6 | 31,50 | 4,31 | 17,93 | 4,47 | 188,50 | 13,45 | 147,67 | 21,99 |
| Molise_colli | | dicoccon | Italy | 6 | 27,73 | 8,80 | 11,13 | 1,35 | 179,10 | 15,28 | 154,55 | 4,47 |
| Cascia_14 | | dicoccon | Italy | 6 | 29,70 | 3,26 | 25,37 | 5,32 | 170,63 | 33,89 | 157,73 | 17,38 |
| Radicondoli | | dicoccon | Italy | 6 | 17,40 | 1,25 | 9,40 | 2,46 | 226,10 | 26,35 | 158,67 | 3,44 |
| Zefiro | | dicoccon | Italy | 6 | 28,83 | 3,15 | 23,63 | 2,20 | 160,50 | 9,41 | 185,00 | 37,00 |

**Tab. S2. Candidate genes identified for all the sequences of the markers mapped in the regions of QTNs.**

| Marker | Code | Chromosome | SNP Location | Gene | Gene location | Gene function | interpro/descript | GO terms | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | BP | MF | CC |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I694,256,760-694,257,795 | ABC transporter-like | AAA+_ATPase | | | ATPase activity ATP binding | |
| | | | | | | Ald_DH/histidinol_DH | | | | |
| | | | | | | methylmalonate-semialdehyde | | oxidation- | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I696,355,533-696,362,953 | Aldehyde dehydrogenase domain | dehydrogenase (acylating) | | reduction process | oxidoreductase activity | |
| | | | | | | Aldose_1/G6P_1-epimerase | | carbohydrate metabolic process | | |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6J137,375,118-137,379,679 | Aldose 1-/Glucose-6-phosphate 1-epimerase | glucose 1-phosphate phosphorylation | | | catalytic activity hydrolase activity | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2J607,601,226-607,602,449 | Alpha/beta hydrolase fold-3 | AB_hydrolase | | | | |
| | | | | | | Alpha1_2_glucosyltferase_Alg | | | | |
| | | | | | | 10 dolichyl-phosphate-glucose- | | dolichol-linked | | |
| | | | | | | glycolipid alpha- | | oligosaccharide | transferase | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2J602,444,766-602,448,443 | Alpha-2-glucosyltransferase Alg10 | glucosyltransferase activity | | biosynthetic process | activity amino acid transmembrane | |
| | | | | | | | AA_transpt_TM integral | | transporter activity | membrane |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I690,930,313-690,931,758 | Amino acid transporter, transmembrane domain | | component of membrane | | | |
| | | | | | | | | | RING-like zinc finger domain | heterochromatin domain |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6J137,874,575-137,877,177 | Aminotransferase-like, plant mobile domain | | AminoTfrase-like_pln_mobile | | binding | |
| | | | | | | | | AMP transport | acetoacetate-CoA ligase activity | |
| | | | | | | | AMP-binding_CS catalytic activity | AMP phosphorylation anaphase-promoting complex-dependent catabolic process | | |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6J135,557,573-135,563,386 | AMP-dependent synthetase/ligase | | | | | |
| | | | | | | | | | anaphase-promoting complex binding | anaphase-promoting complex |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2J607,483,797-607,487,389 | Anaphase-promoting complex subunit 4, WD40 do | | WD40_repeat | | | |
| | | | | | | | ARM-type_fold AP-5 adaptor | | AP-1 adaptor complex binding | AP-5 adaptor complex |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I694,048,655-694,054,006 | AP-5 complex subunit zeta-1 | | complex | | | heterochromatin domain |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I694,990,435-694,992,432 | APO domain | | | | TRAIL binding | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I694,420,113-694,426,360 | B3 DNA binding domain | | B3_DNA-bd | | DNA-binding | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I694,440,854-694,447,076 | B3 DNA binding domain | | | | | |
| | | | | | | | | | RING-like zinc finger domain | |
| | | | | | | | zinc ion binding Znf_B-box | | binding | nucleus |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6J134,947,729-134,949,478 | B-box-type zinc finger / CCT domain | | CCT_domain | | | |
| | | | | | | | Bet_v_I/MLP abscisic acid binding protein phosphatase inhibitor activity abscisic acid-activated signaling pathway | | | |
| | | | | | | | | | | major mitochondrial derivative |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I695,688,695-695,689,617 | Bet v I/Major latex protein | | defense response | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I695,692,974-695,693,871 | Bet v I/Major latex protein | | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I695,732,329-695,733,087 | Bet v I/Major latex protein | | | | | |

| Marker | Code | Chromosome | SNP Location | Gene | Gene location | Gene function | interpro/descript | GO terms | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | BP | MF | CC |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 695,928,158-695,928,501 | Bifunctional inhibitor/plant lipid transfer protein/s | Bifun_inhib/LTP/seed_sf lipid binding | lipid storage intermembrane lipid transfer | lipid transfer activity | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 695,964,161-695,964,504 | Bifunctional inhibitor/plant lipid transfer protein/seed storage helical domain | lipid transport | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 695,992,642-695,992,985 | Bifunctional inhibitor/plant lipid transfer protein/seed storage helical domain | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 696,080,847-696,081,190 | Bifunctional inhibitor/plant lipid transfer protein/seed storage helical domain | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 690,737,314-690,748,846 | BTB/POZ domain | BTB/POZ_dom protein binding | | POZ domain binding | heterochromatin domain |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 691,038,268-691,039,353 | BTB/POZ domain | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 691,187,478-691,190,400 | BTB/POZ domain | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 691,402,062-691,403,135 | BTB/POZ domain | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 691,753,425-691,773,013 | BTB/POZ domain | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 692,111,361-692,112,509 | BTB/POZ domain | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 692,115,276-692,115,806 | BTB/POZ domain | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 692,871,667-692,873,184 | BTB/POZ domain | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 692,893,910-692,895,117 | BTB/POZ domain | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 693,071,730-693,072,986 | BTB/POZ domain | | | | |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6A | 137,523,560-137,534,169 | Calcium-transporting P-type ATPase, N-terminal autoinhibitory domain / Cation-transporting P-type ATPase, N-terminal / | ATPase_P-typ_cation-transptr_C ATP binding calcium transmembrane transporter activity, phosphorylative mechanism | | Calcium transport Ion transport | cation-transporting ATPase complex |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6A | 135,239,729-135,248,399 | CALMODULIN-BINDING PROTEIN60 | CBP60 calmodulin binding | | calmodulin binding | DAPK1-calmodulin complex |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 605,034,659-605,038,359 | CBS domain | CBS_dom protein binding | | SH2 domain binding | heterochromatin domain |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 694,272,868-694,275,994 | CRIB domain | CRIB_dom protein binding | signal transduction | | heterochromatin domain |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 606,509,463-606,527,827 | DNA mismatch repair protein MutS-like, N-termina | DNA_mismatch_repair_MutS-lik_N ATP binding domain_helic_dna2/nam7 helicase-like, aaa-transptr_DNA binding dna2/nam7 helicase transmembrane transporter activity, phosphorylative mechanism | mismatch repair | DNA binding | MutS complex |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 691,007,899-691,012,988 | DNA2/NAM7 helicase, AAA domain / DNA2/NAM7 | DNA2/NAM7 ATP-dependent nuclease | | helicase activity | m-AAA complex |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 605,799,664-605,805,384 | Domain of unknown function DUF4378 | unknown | oxidation-reduction process | oxidoreductase activity | |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6A | 133,755,943-133,761,869 | Fatty acid hydroxylase | Fatty_acid_hydroxylase iron ion binding | fatty acid elongation | fatty acid alpha-hydroxylase activity | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 603,686,802-603,688,917 | F-box domain | F-box_dom protein binding | | F-box domain binding | |

| Marker | Code | Chromosome | SNP Location | Gene | Gene location | Gene function | interpro/descript | GO terms | | |
|---|---|---|---|---|---|---|---|---|---|---|
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| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 695,106,864-695,109,573 | F-box domain | F-box_dom protein binding | | F-box domain binding | |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6 | 133,806,900-133,807,554 | Flavin monooxygenase-like | FAD/NAD-bd_sf flavin adenine dinucleotide binding NADP binding | | flavin adenine dinucleotide binding NADP binding | |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6 | 134,681,939-134,683,491 | Flavin monooxygenase-like | | | | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2 | 607,475,995-607,480,452 | GDSL lipase/esterase | GDSL_lip protein binding hydrolase activity, acting on ester bonds | | lipase activity | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 695,858,915-695,862,524 | GDSL lipase/esterase | GDSL_lip protein binding hydrolase activity, acting on ester bonds | | lipase activity | |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6 | 133305783-133307489 | Glutamine amidotransferase | Glutamine_ami protein binding glutamine metabolic process | glutamine transport and secretion | GMP synthase (glutamine-hydrolyzing) activity | glutathione hydrolase complex |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2 | 607,827,761-607,830,440 | Glutaredoxin | Glutaredoxin_Glu protein binding electron transfer activity protein disulfide oxidoreductase activity cell redox homeostasis | biosynthetic process | arsenate reductase (glutaredoxin) activity glutathione disulfide oxidoreductase activity | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2 | 605,310,908-605,312,618 | Glycoside hydrolase, family 28 | Glyco_hydro_28 polygalacturonase activity carbohydrate metabolic process Cell wall biogenesis/degradation | glycoside transport glycoside metabolic process | polygalacturonase activity | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2 | 602,487,720-602,488,811 | G-patch domain / GC-rich sequence DNA-binding f | G_patch_dom transmembrane transporter activity, phosphorylative mechanism | | DNA binding domain nucleic acid binding | |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6 | 133,372,459-133,375,623 | HD domain | HD_dom protein binding 5'-deoxynucleotidase activity | | SH2 domain binding | heterochromatin domain |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2 | 606,821,901-606,832,906 | HEAT repeat / CLASP N-terminal domain | CBCLASP domain heat repeat binding large substrates such as proteins and nucleic acids | | N-terminal myristoylation domain binding | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 694,054,341-694,061,019 | Homeobox domain | Homeobox_dom lipid binding sequence-specific DNA binding regulation of transcription, DNA-templated | regulation of transcription, DNA-templated | DNA binding | heterochromatin domain |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 691,777,036-691,779,492 | Impact, N-terminal | CBal impact n terminal translational regulator that ensures constant high levels of translation under amino acid starvation | N-terminal protein lipidation | | |

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| Marker | Code | Chromosome | SNP Location | Gene | Gene location | Gene function | interpro/descript | BP | MF | CC |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 690,907,263-690,909,532 | IQ motif, EF-hand binding site / Domain of unknow | may be involved in cooperative interactions with calmodulins or calmodulin-like proteins, and may associate with nucleic acids and regulate gene expression at the transcriptional or post-transcriptional level | | calcium ion binding | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 607,820,263-607,827,519 | Isopropylmalate dehydrogenase-like domain | IsoCit/isopropylmalate_DH_CS isocitrate dehydrogenase (NADP+) activity magnesium | isocitrate metabolic process tricarboxylic acid cycle | ion binding | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 695,594,529-695,595,176 | Late embryogenesis abundant protein, LEA_2 subg | LEA_embry_late-transptr_binding | | | integral component of membrane |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 603,557,030-603,561,199 | Leucine-rich repeat-containing N-terminal, plant-t | Leu-rich_rpt Protein kinase domain-containing protein | nucleotide-binding domain, leucine rich repeat containing receptor signaling pathway N-terminal peptidyl-leucine methylation | ATP binding protein kinase activity | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 604,750,242-604,754,542 | Leucine-rich repeat-containing N-terminal, plant-type | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 690,962,852-690,963,880 | Leucine-rich repeat-containing N-terminal, plant-t | Leu-rich_rpt Protein kinase domain-containing protein | nucleotide-binding domain, leucine rich repeat containing receptor signaling pathway N-terminal peptidyl-leucine methylation | ATP binding protein kinase activity | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 690,967,351-690,968,786 | Leucine-rich repeat-containing N-terminal, plant-type | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 695,372,688-695,376,293 | Leucine-rich repeat-containing N-terminal, plant-type / Leucine-rich repeat / Protein kinase domain | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 690,785,326-690,786,411 | MATH/TRAF domain | protein binding may play an important role in plant development and stress tolerance | TRAF-mediated signal transduction | MATH domain binding zf-TRAF domain binding | heterochromatin domain |

| Marker | Code | Chromosome | SNP Location | Gene | Gene location | Gene function | interpro/descript | GO terms | | |
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| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 691,047,457-691,056,107 | MATH/TRAF domain / BTB/POZ domain | | | MATH domain binding zf-TRAF domain binding POZ domain binding | heterochromatin domain |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 692,161,873-692,163,580 | MATH/TRAF domain / BTB/POZ domain | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 693,095,105-693,096,262 | MATH/TRAF domain / BTB/POZ domain | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 693,153,766-693,154,991 | MATH/TRAF domain / BTB/POZ domain | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 693,304,708-693,305,760 | MATH/TRAF domain / BTB/POZ domain | | | | |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6A | 137,337,421-137,338,660 | Myb/SANT-like domain | appears in several proteins annotated as transposon proteins | | RING-like zinc finger domain binding | Myb complex heterochromatin domain |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 691,809,625-691,810,897 | Myc-type, basic helix-loop-helix (bHLH) domain | transmembrane transporter activity, phosphorylative mechanism protein dimerization activity | | bHLH transcription factor binding | lateral loop plasma membrane |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6A | 137,632,895-137,633,217 | NADH-Ubiquinone oxidoreductase (complex I), cha | NADHpl_OxRdtase_5_subgr nadh transmembrane transporter | mitochondrial electron transport, NADH to ubiquinone | NADH dehydrogenase (ubiquinone) activity | respiratory chain complex I |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 605,165,521-605,167,463 | NB-ARC | LRR_dom_sf NB-ARC ADP binding | | disruption by symbiont of host cell PML body | host cell PML body |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 605,126,841-605,128,592 | NB-ARC domain-containing protein. | ADP-binding, Belongs to the disease resistance NB-LRR family. | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 692,071,295-692,073,993 | O-methyltransferase domain | O_MeTrfase_dom o methyltransferase activity protein dimerization activity methylation | | O-methyltransferase activity | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 692,163,867-692,167,946 | PAS fold-4 / Signal transduction histidine kinase, dimerisation/phosphoacceptor domain / Histidine kinase/HSP90-like ATPase | HATPase_C Histidine kinase domain-containing protein phosphorelay sensor kinase activity | activation of protein histidine kinase activity | protein histidine kinase binding | protein histidine kinase complex |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 695,587,929-695,590,007 | Pentatricopeptide repeat | Pentatricopeptide_rep protein binding | RNA metabolism | ankyrin repeat binding | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 696,627,161-696,627,619 | Pentatricopeptide repeat | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 695,889,106-695,891,854 | Pentatricopeptide repeat | | | | |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6A | 135,444,923-135,446,233 | Petal formation-expressed | unknown | petal formation | | integral component of membrane |

| Marker | Code | Chromosome | SNP Location | Gene | Gene location | Gene function | interpro/descript | GO terms | | |
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| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 691,453,016-691,454,872 | Phospholipase A2 | PLipase_A2 calcium ion binding | lipid catabolic process phospholipid metabolic process activation of phospholipase A2 activity | phospholipase A2 activity | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 602,477,382-602,481,614 | Phosphoribosyltransferase domain | Phosphoribosyltransferase_dom protein binding | nucleoside metabolic process | adenine phosphoribosyltransferase activity | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 603,548,387-603,550,808 | PMR5 N-terminal domain | PMR5_N_dom have Acyl esterase activity and predicted to modify cell-surface biopolymers such as glycans and glycoproteins | N-terminal protein formylation N-terminal protein lipidation | N-terminal myristoylation domain binding | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 607,511,599-607,514,396 | PMR5 N-terminal domain / PC-Esterase | | N-terminal protein lipidation | N-terminal myristoylation domain binding | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 607,908,192-607,910,525 | Proliferating cell nuclear antigen, PCNA, N-termina | Pr_cel_nuc_antig DNA binding DNA polymerase processivity factor activity | DNA clamp unloading regulation of DNA replication | | PCNA complex |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 691,356,058-691,357,661 | Proteasomal ATPase OB C-terminal domain / ATPase, AAA-type, core / AAA ATPase, AAA+ lid domain | AAA+_ATPase | | ATP binding | membrane |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 691,427,986-691,429,372 | Proteasomal ATPase OB C-terminal domain / ATPase, AAA-type, core / AAA ATPase, AAA+ lid domain | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 695,579,288-695,582,940 | Proteasomal ATPase OB C-terminal domain / ATPase, AAA-type, core / AAA ATPase, AAA+ lid domain | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 695,579,288-695,582,940 | Proteasomal ATPase OB C-terminal domain / ATPase, AAA-type, core / AAA ATPase, AAA+ lid domain | | | | |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6A | 133,414,170-133,419,353 | Protein kinase domain | Prot_kinase_dom | | protein kinase activity | |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6A | 135,053,305-135,067,604 | Protein kinase domain | | | | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 606,674,389-606,675,908 | Protein LOW PSII ACCUMULATION 1-like | integral component of membrane chaperone required for efficient photosystem II (PSII) assembly. It binds to psbA during de novo biogenesis of PSII | pigment accumulation | ubiquitin-like protein binding | membrane |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 603,562,212-603,563,873 | Protein of unknown function DUF247, plant | unknown | plant organ morphogenesis | | plant cell papilla |

| Marker | Code | Chromosome | SNP Location | Gene | Gene location | Gene function | interpro/descript | GO terms | | |
|---|---|---|---|---|---|---|---|---|---|---|
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| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 602,797,239-602,802,183 | Pyruvate/Phosphoenolpyruvate kinase-like domain | Pyrv/PenolPyrv_Kinase-like_dom catalytic activity Pyruvate kinase controls the exit from the glysolysis pathway | phosphoenolpyruvate transport | pyruvate kinase activity | pyruvate kinase complex |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6A | 136,163,088-136,163,438 | Reversibly glycosylated polypeptide family | cell wall establishment and plant development | | glycosylated region protein binding | membrane |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 605,915,933-605,916,403 | Ribosomal protein S7 domain | translation small ribosomal subunit | ribosomal skipping ribosome disassembly | structural constituent of ribosome | ribosomal subunit |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2B | 695,111,907-695,112,860 | Ribosome-inactivating protein | Ribosome_inactivat_prot defense response | negative regulation of translation | rRNA N-glycosylase activity | ribosome |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2B | 695,115,589-695,116,562 | Ribosome-inactivating protein | | | | |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6A | 134,170,916-134,193,026 | RNA polymerase, beta subunit, protrusion / RNA p | RNA_pol_bsu_protrusion DNA binding | transcription, DNA-templated | RNA polymerase activity | RNA polymerase complex |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2B | 692,460,045-692,464,110 | RNA recognition motif domain | | phagocytosis, recognition | nucleic acid binding RNA binding | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 602,807,054-602,812,462 | RNA recognition motif domain / Polyadenylate-binding protein/Hyperplastic disc protein | | | nucleic acid binding RNA binding | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 605,164,670-605,172,302 | Rx, N-terminal | Plant defense The N-terminal coiled-coil domain of RX has been shown to interact with RanGAP2, which is a necessary co-factor in the resistance response | N-terminal protein formylation N-terminal protein lipidation | DNA binding | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 605,128,979-605,129,740 | Rx, N-terminal / NB-ARC | | | | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 605,398,621-605,399,699 | Rx, N-terminal / NB-ARC | | | | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 606,676,920-606,679,840 | Rx, N-terminal / NB-ARC | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2B | 693,321,417-693,326,149 | Rx, N-terminal / NB-ARC | Plant defense The N-terminal coiled-coil domain of RX has been shown to interact with RanGAP2, which is a necessary co-factor in the resistance response | N-terminal protein formylation N-terminal protein lipidation | DNA binding | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2B | 693,447,357-693,452,488 | Rx, N-terminal / NB-ARC | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2B | 693,723,708-693,728,415 | Rx, N-terminal / NB-ARC | | | | |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6A | 136,541,404-136,544,531 | SBP domain | SBP zinc finger | | SH2 domain binding DNA binding metal ion binding | heterochromatin domain |

| Marker | Code | Chromosome | SNP Location | Gene | Gene location | Gene function | interpro/descript | GO terms | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | BP | MF | CC |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 604,860,350-604,865,276 | Serine incorporator/TMS membrane protein | integral component of membrane | membrane protein proteolysis | protein-serine epimerase activity | membrane protein complex |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 608,043,925-608,048,130 | Serine-threonine/tyrosine-protein kinase, catalytic | Prot_kinase_dom ATP binding protein kinase activity | protein phosphorylation | protein serine/threonine /tyrosine kinase activity | serine/threonine protein kinase complex |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 602,439,030-602,444,330 | Serine-threonine/tyrosine-protein kinase, catalytic | domain | | | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 608,032,557-608,036,387 | Ssl1-like / TFIIH C1-like domain | transcription factor TFIIH core complex | nucleotide-excision repair transcription, DNA-templated | RING-like zinc finger domain binding | nucleolus-like body |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 603,082,948-603,086,193 | Sugar phosphate transporter domain | Sugar_P_trans_dom | cellular response to sugar-phosphate stress | sugar transmembrane transporter activity | integral component of membrane |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2B | 694,532,159-694,533,010 | SUI1 domain | SUI_dom protein binding | translational initiation | translation initiation factor activity SH2 domain binding | heterochromatin domain |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2B | 696,668,269-696,673,438 | Terpene synthase, N-terminal domain / Terpene synthase, metal-binding domain | Terpene_synth_N magnesium | terpene metabolic | ion binding terpene synthase | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 602,448,544-602,452,363 | Trafficking protein particle complex subunit 2 | Longin-like_dom_sf | protein-DNA complex subunit organization endoplasmic reticulum to Golgi vesicle-mediated transport | | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 603,523,617-603,524,613 | Transcription factor CBF/NF-Y/archaeal histone do | CBFA_NFYB_domain | sequence-specific DNA binding regulation of transcription, DNA-templated | protein heterodimerization activity | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 606,711,012-606,712,790 | Transferase | transferring acyl groups other than amino-acyl groups | regulation of transferase activity | transferase activity | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 606,812,971-606,814,768 | Transferase | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2B | 691,415,951-691,417,582 | Transferase | transferring acyl groups other than amino-acyl groups | regulation of transferase activity | transferase activity | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 607,875,093-607,876,801 | Transmembrane protein GPR107/GPR108-like | CBGPR107/GPR108-like transmembrane protein | protein transmembrane transport | ubiquitin-like protein binding | integral component of membrane |

| | | | | | | | GO terms | | |
|---|---|---|---|---|---|---|---|---|---|
| Marker | Code | Chromosome | SNP Location | Gene | Gene location | Gene function | interpro/descript | BP | MF | CC |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 695,449,352-695,451,719 | U box domain / Armadillo | protein modification - function in various processes, including intracellular signalling and cytoskeletal regulation | | armadillo repeat domain binding ubiquitin-protein transferase activity | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2/ | 607,481,662-607,483,729 | UbiE/COQ5 methyltransferase | 2-phytyl-1,4-naphthoquinone | methylation, phylloquinone biosynthetic process | methyltransferase activity | methyltransferase complex |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2/ | 604,748,338-604,750,173 | UDP-glucuronosyl/UDP-glucosyltransferase | UDP_glucos_trans UDP_glycos_trans_CS | | transferase activity, transferring hexosyl groups | membrane |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 694,943,732-694,945,696 | UDP-glucuronosyl/UDP-glucosyltransferase | UDP_glucos_trans UDP_glycos_trans_CS | | transferase activity, transferring hexosyl groups | membrane |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6/ | 134,544,449-134,547,770 | Vacuolar import/degradation Vid27, C-terminal | unknown possibly regulates import of fructose-1,6-bisphosphatase into Vacuolar Import and Degradation (Vid) vesicles and is not essential for proteasome-dependent degradation of fructose-1,6-bisphosphatase | | C-terminal protein methylation amino-terminal | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 696,677,125-696,684,238 | Vacuolar protein sorting-associated protein 13, N-t | unknown may act as a negative regulator of cell growth | N-terminal protein lipidation | vacuolar sorting propeptide binding | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 696,371,508-696,376,089 | WIYLD domain / Pre-SET domain / SET domain | histone-lysine N-methyltransferase activity zinc ion binding involved in epigenetic control of gene function | nucleosome assembly | DNA binding | heterochromatin domain |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2/ | 604,858,358-604,859,536 | Zinc finger, GRF-type | zinc ion binding likely involved in nucleic acid binding | | zinc finger domain binding | integral component of membrane |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2I | 695,517,441-695,517,977 | Zinc finger, RING-type | probably involved in mediating protein-protein interactions | | RING-like zinc finger domain binding | integral component of membrane |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2/ | 607,995,741-608,000,800 | Zinc finger, RING-type / von Willebrand factor, typ | unknown Proteins that incorporate vWF domains participate in numerous biological events (e.g. cell adhesion, migration, homing, pattern formation, and signal transduction), involving interaction with a large array of ligands | | RING-like zinc finger domain binding | integral component of membrane |

| Marker | Code | Chromosome | SNP Location | Gene | Gene location | Gene function | interpro/descript | GO terms | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | BP | MF | CC |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 602,650,886-602,653,188 | Zinc finger, RING-type / von Willebrand factor, type A / VWA-Hint protein, Vwaint domain | | | RING-like zinc finger domain binding | integral component of membrane |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 602,482,297-602,483,176 | unknown | | | | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 602,687,991-602,688,847 | unknown | | | | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 602,717,923-602,718,740 | unknown | | | | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 602,765,980-602,766,809 | unknown | | | | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 603,368,804-603,370,887 | unknown | | | | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 603,374,752-603,376,894 | unknown | | | | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 603,545,891-603,547,668 | unknown | | | | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 603,855,475-603,857,096 | unknown | | | | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 604,494,566-604,495,201 | unknown | | | | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 605,115,456-605,115,707 | unknown | | | | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 605,209,183-605,210,227 | unknown | | | | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 605,336,239-605,337,360 | unknown | | | | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 605,400,017-605,402,122 | unknown | | | | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 605,592,205-605,593,188 | unknown | | | | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 607,517,820-607,521,203 | unknown | | | | |
| AX-94536561 | BA00857358 | 2A | 605184812 | TraesCS2A | 607,597,019-607,600,759 | unknown | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2B | 691,451,450-691,452,939 | unknown | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2B | 691,780,485-691,780,943 | unknown | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2B | 692,174,074-692,176,365 | unknown | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2B | 692,710,731-692,714,121 | unknown | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2B | 692,956,329-692,956,877 | unknown | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2B | 693,346,300-693,346,833 | unknown | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2B | 693,885,306-693,896,693 | unknown | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2B | 694,266,427-694,267,446 | unknown | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2B | 694,541,612-694,544,550 | unknown | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2B | 695,169,783-695,172,112 | unknown | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2B | 695,508,064-695,516,036 | unknown | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2B | 695,583,255-695,583,938 | unknown | | | | |
| AX-95019471 | BA00784407 | 2B | 693730612 | TraesCS2B | 695,590,207-695,593,740 | unknown | | | | |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6A | 133,824,894-133,826,020 | unknown | | | | |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6A | 134,202,119-134,203,501 | unknown | | | | |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6A | 134,551,940-134,552,236 | unknown | | | | |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6A | 134,935,079-134,935,582 | unknown | | | | |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6A | 135,424,034-135,424,652 | unknown | | | | |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6A | 135,433,120-135,433,942 | unknown | | | | |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6A | 135,435,823-135,438,904 | unknown | | | | |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6A | 136,654,576-136,660,258 | unknown | | | | |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6A | 136,947,273-136,948,640 | unknown | | | | |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6A | 137,380,238-137,381,136 | unknown | | | | |

| | | | | | | | | GO terms | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Marker | Code | Chromosome | SNP Location | Gene | Gene location | Gene function | interpro/descript | BP | MF | CC |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6 | 137,380,238-137,381,136 | unknown | | | | |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6 | 137,392,432-137,393,238 | unknown | | | | |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6 | 137,392,432-137,393,238 | unknown | | | | |
| AX-94438966 | BA00318879 | 6A | 135245932 | TraesCS6 | 137,873,483-137,873,746 | unknown | | | | |

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# CHAPTER 5

# Brief conclusion

The work carried out and described in chapters 2, 3 and 4 represents the chronological phases of a research addressed to an important and up-to-date topic by exploring the genetic diversity between *Triticum turgidum* accessions and detecting QTLs for mycorrhizal colonization.

Most of the studies focusing on the genetic diversity study between grain accessions, have analyzed separately the subspecies, including ssp. *durum* [1,2], ssp. *dicoccum* [3,4], ssp. *polonicum* [5] and ssp. *dicoccoides* [6], while few are the works carried out on *Triticum turgidum* wheats [7,8]. In this study, SNPs markers provided an accurate picture of the population structure within tetraploid wheat collections, which is an essential information for the design of association analyses. Probably, an higher number of accessions could allow as to better understand the influence of origin on the genetic variability between wheats. Neverthess, a strategic platform for the future association mapping studies and for the study of phenotypic traits related to *T. turgidum* wheats of the Mediterranean Region is given.

The genome-wide association analyisis, conducted on 127 genotypes previously characterized and aimed at QTLs detection for mycorrhizal colonization, explores a new topic, not well discussed by research. In addition, the work represents the study which considers the highest number of individuals. As reviewed in chapter 3, AMF improve plant performance by providing ecosystem services such as enhancing plant tolerance to biotic and abiotic stresses, supply of nutrients, increase of water use efficiency and the change of physico-chemical properties of soils. The knowledge of AM fungi-associated QTLs provided in this thesis might be used in marker-assisted breeding of durum wheat and thereby enhance plant performance under stressful environmental conditions. However, a future development of this study might repeat the experiment in the field, looking for QTLs detection and comparation with those founded in the lab.

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# APPENDIX

## 6.1 Concealed metabolic reprogramming induced by different herbicides in tomato

### 6.1.1 Introduction

Agrochemicals are a category of substances with diverse chemical characteristics that are widely used in the field against pests and weeds, as phytoregulators, or biostimulants. Within this category, an important class of compounds is represented by herbicides, which are normally used in agriculture for the control of weeds. The mechanism of action of herbicides is well known. Most of the literature has focused on environmental fate and residues in edible parts, whereas much less information is available regarding their impact on plant metabolism and biochemical processes, which in turn affects plant productivity and quality of fruits. Nonetheless, preliminary evidence has suggested that their application may result in stress of crop plants, even when selective herbicides are used on tolerant crops and according to good agricultural practice. In fact, abiotic stresses (including the application of xenobiotics) can be associated with the activation of stress-defense pathways, involving the modulation of the plant hormone balance, such as changes in abscisic acid (ABA), brassinosteroids and auxins [1], and salicylates and jasmonates [2]. Once released in the environment, herbicides can reach the plant directly (for selective molecules) or indirectly (for non-selective compounds). Within this framework, the active substance is partly retained by the soil, partly evaporates into the atmosphere, and is partly conveyed to the shallow waterways [3]. Its persistence depends on several factors, including degradability, half-life time, and leaching; the available fraction can eventually be absorbed by the plant roots. The current literature suggests that availability is correlated mainly with the lipophilicity of the chemical, whereas compounds with intermediate polarity, weak acids, and amphiphilic xenobiotics are more easily transported in the hydraulic system of the plant [4]. To date, most of the literature on pesticides in plants relates to the study of their mode of action, detoxification and metabolization, or their residues in edible portions. However, an increasing number of clues suggest that xenobiotics may interfere with plant functions and metabolism, causing stress even at concentrations below the levels at which Journal Preproof 5 stress symptoms are evident at the phenotype level [5,6]. Notably, the effect of herbicides on plant metabolism is worth to be investigated also

when the herbicides, such as pre-emergence herbicides and non-selective substances, are applied to the soil. In the understanding of plant responses to agrochemicals, metabolomics can be of great help since it provides a complete set of metabolites, typically low-molecularweight compounds, with a holistic perspective [7]. Indeed, metabolomics allows identifying molecular phenotypes of plants in response to environmental stresses, in order to find particular patterns associated with tolerance or adaptation [8,9]. Considering the wide diversity of herbicides, the specific effect of an active substance on plant metabolism is compound or class dependent. Glyphosate (Gly) and metribuzin (MB) are among the most commonly used herbicides. MB is a triazinone pre- and post-emergent selective herbicide that controls a range of dicot and monocot weed species in several crops. MB's mechanism of action involves the inhibition of photosynthesis at the level of Hill's reaction [10]. As a PSII-inhibiting herbicide, MB inhibits photosynthesis by blocking the electron transport from QA to QB, thus leading to photooxidation of lipid and chlorophyll and, consequently, plant death [11]. On the other hand, Gly is a non-selective and non-residual herbicide acting on manganese chelation, a cofactor of the enzyme 5enolpyruvylshikimate-3-phosphate (EPSP) synthase of the shikimate pathway, which is responsible for the biosynthesis of aromatic amino acids [12]. Other herbicides, such as the selective sulphonylurea rimsulfuron (RM), influence the enzyme acetolactate synthase (ALS), therefore affecting the biosynthesis of the branched-chain amino acids. Among others, the nonselective herbicide pelargonic acid (PA—the trivial name of nonanoic acid) is gaining popularity in both conventional and organic agriculture because of its broad-spectrum contact action. Starting from this diversity of mechanisms, the present study aims to shed light on the effects of two widely important herbicides, MB and Gly, and two others, RM and PA, on the primary and secondary metabolism of tomato (*Solanum lycopersicum* L.) plants. This plant represents a good model because Journal Pre-proof 6 it is one of the most common vegetables worldwide, has a diverse secondary metabolism, and has fruits that are an important source of functional ingredients in the human diet [13]. Together with a better comprehension of the hidden biochemical perturbations induced by herbicides in plants, these results can pave the way toward a holistic perspective in environmental sciences, in the framework of revisited

agronomic practices, and in terms of both productivity and quality of raw agricultural commodities.

### 6.1.2 Material and methods

*Growth conditions, plant material, and experimental design*

The experiment was carried out in an experimental greenhouse situated at Università Cattolica del Sacro Cuore of Piacenza, Italy. Seedlings of tomato cv. Heinz 3402 at the four-true-leaf stage were transplanted into pots containing a commercial loam (Vigorplant 5 stelle, pH = 6.1, EC 1.1 dS m-1 ) in the middle of April. Five seedlings per pot were placed in ten 40-cm pots, and two seedlings per pot in twenty-five 30-cm pots. Smaller pots were used for the test on plant metabolism, and the life cycle was stopped at 10 days after herbicide application, when leaves were harvested for subsequent analysis. However, bigger pots were used for investigations of fruits, and leaves were not harvested following treatment; the pots were watered with 2 L of water every 3–4 days. 2 L of basic nutrient solution (13 mmol L-1 NO3N, 1 mmol L-1 NH4-N, 1.75 mmol L-1 S, 1.5 mmol L-1 P, 5 mmol L-1 K, 4.5 mmol L-1 Ca, 2 mmol L-1 Mg, 1 mmol L-1 Na, 1 mmol L-1 Cl, 20 µmol L1 Fe, 9 µmol L-1 Mn, 0.3 µmol L-1 Cu, 1.6 µmol L-1 Zn, 20 µmol L-1 B, and 0.3 µmol L-1 Mo), with an electrical conductivity of 2.0 dS m-1 , was applied at 45 and 90 days after transplantation to pots used for investigations of fruits. The treatments were arranged in a one-factor, completely randomized design, with 10 plants per treatment (5 pots) for the investigation of metabolic changes and with 20 plants per treatment (4 pots) Journal Preproof 7 for the analysis of berries. An untreated control was used for investigations of metabolic changes (10 plants), and another for analyses of berries (20 plants). Selection and application of the herbicides Two selective herbicides, MB and RM, and two non-selective herbicides, Gly and PA, were used. Besides selectivity, these herbicides were chosen to account for different mechanisms of action. MB is a triazine with a mechanism of action involving the inhibition of photosynthesis, whereas the sulfonylurea RM inhibits the synthesis of the branched-chain amino acids [14]. Meanwhile, Gly affects the shikimate pathway and, therefore, the biosynthesis of aromatic amino acids. PA goes through the cuticle and cell membranes, reducing the

internal pH of the plant cells and causing the pools of cellular ATP and glucose-6-phosphate to decline [15]. The selected herbicides were used as commercial formulations: Medor 35 class (35% metribuzin, WG formulated, Gowan), Executive (25% rimsulfuron, DF formulated, DuPont), Roundup platinum (480 g L-1 glyphosate, SC formulated, Monsanto), and Beloukha (680 g L -1 pelargonic acid, EC formulated, Belchim crop protection). Spray solutions were prepared in tap water according to the manufacturers' instructions provided by the label recommendations. Each spray solution was applied through a manual sprayer (about 25 mL per pot) carefully avoiding contaminations. Control pots were treated with tap water. The treatments were administered at the five-true-leaf stage, one week after transplanting, in late April.

*Sample preparation, antioxidant capacity, and total phenolics*

Leaf samples were harvested at 10 days after the application of the herbicides. Five older and five younger leaves were picked up from each plant, immediately quenched in liquid nitrogen, and then crushed by mortar and pestle in liquid nitrogen. Ground samples (1.0 g) were extracted in 10 mL of a mixture of methanol/acetonitrile/water 1:1:1 (v/v) by using an IKA Ultra-Turrax T18 for Journal Pre-proof 8 comminution. The suspensions were centrifuged (8000 x g), and the supernatants were collected and transferred into a vial for analysis. Analyses of tomato berries were carried out only for controls and plants treated with RM, MB, and PA, since Glytreated plants did not survive the treatment. Berries were harvested scalarly, once they reached the full ripening, during the whole production cycle of the plants. The minimum time between herbicide application and harvest was 96 days. Number of berries and weight were recorded, and then fruits were immediately frozen at -18 °C. Subsequently, they were homogenized, and five replicates of chopped tomato were taken per treatment, including the control. Thereafter, pH and total sugar level were measured using a pH meter and a refractometer, respectively. Finally, the samples were extracted as previously reported for leaves. Total phenolic content (TPC) was determined using Folin-Ciocalteu reagent, while antioxidant activity was measured by ferric reducing antioxidant power (FRAP) analysis, as previously reported [16]. These analyses were carried out in fruits only. With this aim, three replicates

(3 g) from each pooled fruit sample were extracted in 30 mL of 80% methanol (1% formic acid) and centrifuged (6500 rpm for 10 min, 4 °C). Additionally, nitrogen and carbon contents were measured through Dumas combustion using an elemental analyzer (Elemental vario MAX CN, Langenselbold, Germany), and the results were expressed as %N and %C, respectively, in mg per 100 mg of fruit.

*Untargeted screening of metabolites*

The screening of plant metabolites was carried out according to an untargeted metabolomics approach on a quadrupole-time-of-flight (Q-TOF) mass spectrometer coupled to a UHPLC chromatographic system (UHPLC/Q-TOF), as previously reported [17]. A 1290 LC system, equipped with a binary pump and an electrospray ionization source, was coupled to a G6550 Q-TOF mass spectrometer Journal Pre-proof 9 (Agilent Technologies Santa Clara, CA, USA). Briefly, a reverse phase chromatographic separation was performed using a C18 column and a binary gradient consisting of 5% to 90% methanol in water (in 30 min). The injection volume was 3 L, and the flow rate was 200 L/min. The mass spectrometer was run in the positive polarity and SCAN mode (range of 100–1600 m/z in the extended dynamic range setting). Compound identification was based on both monoisotopic accurate mass and isotope pattern (accurate spacing and isotope ratio) and expressed as an overall identification score. Annotations were done using Profinder B.07 software (Agilent technologies) and the database imported from PlantCyc 9.6 (Plant Metabolic Network, http://www.plantcyc.org). However, the Phenol-Explorer 3.6 database (http://phenol-explorer.eu) was used to annotate phenolic compounds from fruit extracts. Compounds abundance profiles were expressed on fresh weight basis.

*Statistical analysis*

Interpretation and statistical analysis of the metabolomics analyses were carried out using Mass Profiler Professional 12.6 (Agilent Technologies). The abundance of identified compounds was log2 transformed and normalized at

the 75th percentile and then baselined against the median of each compound in all samples [18]. Thereafter, unsupervised cluster analysis (Euclidean distance, Ward's linkage rule) in leaves and partial least squares discriminant analysis (PLS-DA) in fruits were performed to describe similarities/dissimilarities across treatments. Finally, Volcano Plot analysis was performed ($p < 0.05$, Bonferroni testing correction; fold change > 1.5), and the output was exported to the PlantCyc Pathway Tool software [19] to highlight the principal metabolic pathways and processes involved in the tomato plants' response to treatments. Differential phenolic compounds in fruits were selected by Volcano Plot analysis ($p < 0.05$, Bonferroni testing correction; fold change > 2). The results obtained from fruit characterization were statistically analyzed by one-way ANOVA analysis ($p < 0.05$) followed by Tukey's HSD post hoc test by using SPSS Statistics 25 software.

### 6.1.3 Results

*Metabolic profiling of tomato plants by UHPLC/QTOF-MS UHPLC/QTOF- MS*

Untargeted metabolomics analysis was carried out in order to understand the effect(s) of the tested pesticides at the biochemical level in tomato plants. Overall, more than 3,600 compounds were annotated and used for further statistical analysis. The entire list of metabolites annotated in our samples is provided as supplementary material, together with individual abundances and composite mass spectra (Supplementary Table S1). A foldchange-based hierarchical clustering, which is an unsupervised multivariate approach, was first performed to describe similarities and distances across treatments. The analysis highlighted two superclusters, in which RM and MB were grouped apart, while PA and Gly were closer to the non-treated plants (Fig. 1). These results pointed out comparable metabolic profiles in plants treated with RM and MB, since an overlapping between samples of these treatments could be observed. The other main cluster included PA and Gly (each of them having distinct metabolic profiles), together with the control. Consequently, this analysis suggested that compared to RM and MB, these latter herbicide treatments induced a milder molecular reprogramming (Fig. 1). Thereafter, discriminant compounds were identified through Volcano Plot analysis ($p <$

0.05; fold change > 1.5) and imported into the PlantCyc Pathway Tool software. Overall, 247 compounds involved in the tomato plants' response to the treatments were identified. Differential compounds, classified based on their functional class, are summarized in Table 1, whereas the complete list is provided as supplementary material (Supplementary Table S2). Therein, a diversity of metabolites, including mainly amino acids, lipids, secondary metabolites, and nucleoside- and nucleotide-related compounds, was represented. More detailed information about the chemical classes of the differential metabolites, as provided by the PlantCyc Pathway Tool, is provided in Fig. 2A. Among the amino acid compounds, the leucine precursor 4-methyl-2oxopentanoate was up-accumulated in MB- and Journal Pre-proof 11 RMtreated plants, whereas glutamine was increased, and methionine decreased in all treatments, compared to the controls. Regarding secondary metabolism, the phenylpropanoid biosynthesis was repressed as a common response to Gly, MB, and RM (Fig. 2B). In addition, 2-O-caffeoylglucarate and the indole glucosinolate 4-hydroxyglucobrassicin derived from tryptophan were strongly down-accumulated by MB and RM. In contrast, glucosinolate-related compounds derived from methionine were increased: the aliphatic glucosinolate 5-(methylsulfinyl)pentylglucosinolate was upaccumulated after RM treatment, while 8-(methylsulfinyl)octylglucosinolate was strongly increased in all treatments. Moreover, Nhydroxy-4-(methylsulfanyl)butimidothioate, a compound involved in glucosinolate biosynthesis from homomethionine [20], was upaccumulated in all treatments. On the other hand, isoprenoids and other compounds involved in the mevalonic acid (MVA) biosynthetic pathway were represented in this study. (R)-mevalonate-5-phosphate was largely downaccumulated by RM and MB, while presqualene diphosphate was strongly down-accumulated only by RM. In fact, the diterpenes ent-7 hydroxykaur-16-en-19-oate and ent-kaurenol, which are precursors of gibberellins, were up-accumulated in the treated plants. In agreement with this, the gibberellin GA34, resulting from the degradation of active gibberellins, was decreased in all treated plants except for Gly. Concerning other hormones, the jasmonate precursors (3-oxo-2-(cis-2'-pentenyl)cyclopentane-1-(3R-hydroxyoctanoyl)-CoA and 3-oxo-2-(cis-2'pentenyl)cyclopentane1-(E-octa-2-enoyl)-CoA) were found to be decreased by the treatments. Only slight variations could be found for other hormones,

with the cytokinin dihydrozeatin being decreased by Gly and PA and with the ABA inactivation compound hydroxy-ABA being accumulated in all treatments but Gly. Regarding lipids, a generalized increase in monoglycerides could be detected in all treatments except Gly, while oxo- and hydroxy-derivatives of acyl-CoA up-accumulated in all treatments. Together with these oxidized forms of fatty acids, other metabolites involved in the redox balance and being modulated by the treatments were tocotrienol (increased in all treatments), ascorbate and Journal Pre-proof 12 dehydroascorbic acid (both decreased in the treated plants), and baicalein (a redox detoxification compound, being down-accumulated in all treatments but PA).

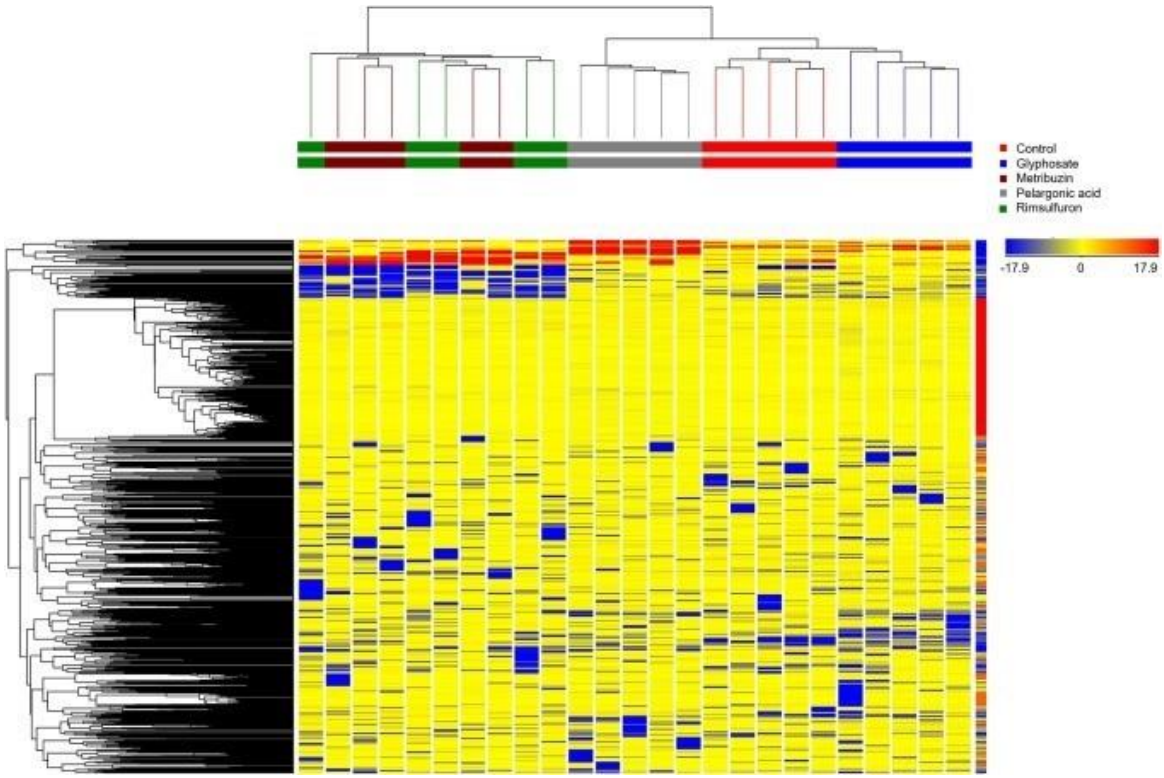


**Fig. 1. Unsupervised hierarchical cluster analysis (Euclidean distance; linkage rule: Ward) of tomato leaf chemical profiles after herbicide application.** Metabolites were obtained by UHPLC-ESI/QTOF-MS untargeted analysis, and their intensities were used to create the fold-change heatmap provided here.

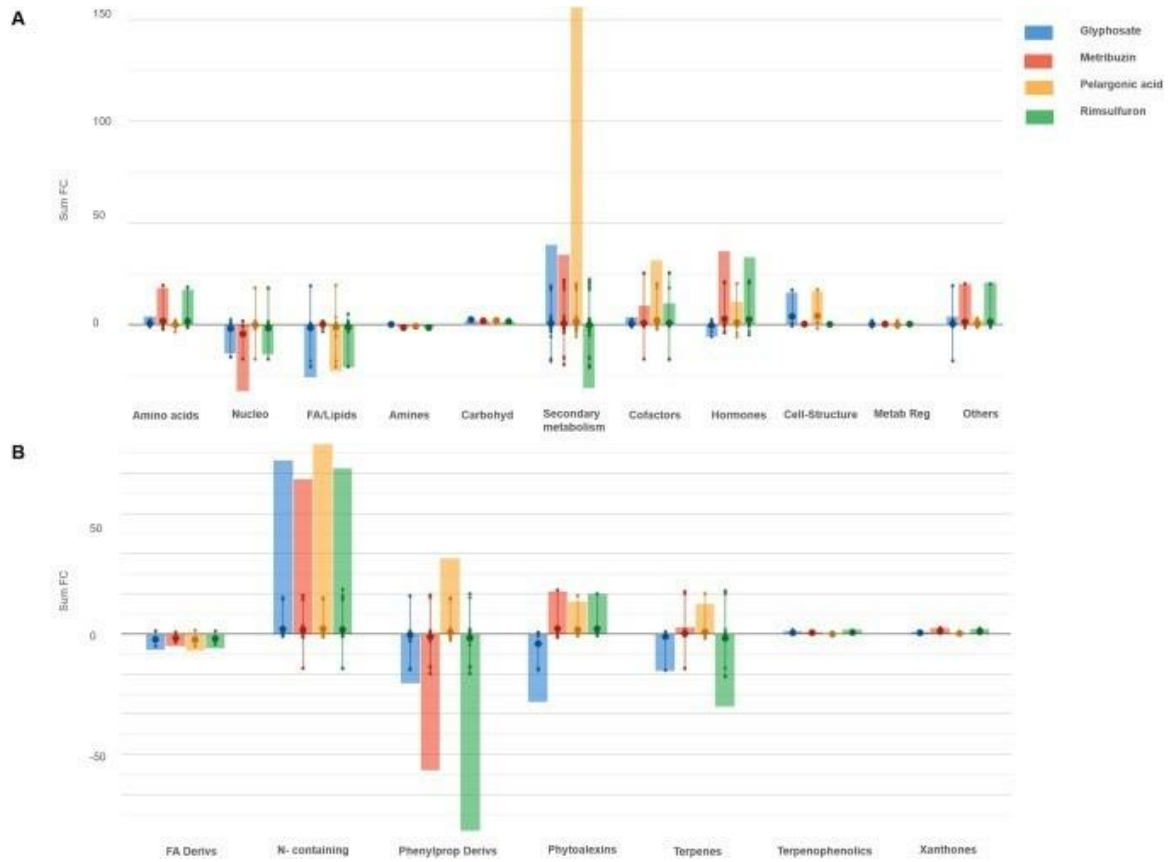


**Fig. 2. Metabolic processes (A) and secondary metabolite biosynthesis (B) are impaired in tomato leaves as a result of the herbicide treatments.** The metabolomics dataset produced through UHPLC-ESI/QTOF-MS was subjected to Volcano Plot analysis ($p < 0.05$, fold change >= 1.5), and differential metabolites were loaded into the PlantCyc Pathway Tool (https://www.plantcyc.org/). The x-axis represents each set of metabolic subcategories, while the y-axis corresponds to the cumulative log fold change (FC). The large dots represent the average (mean) of all FCs for the different metabolites in the class, while the small dots represent the individual log FC.

*Effect of the herbicides on yield components*

Despite a decrease in the number of fruits per pot in the treated plants, a significant increase in the fresh weight (FW) of berries was observed after MB and PA application, with respect to controls (19.5% and 25.2%, respectively; Table 2). However, no significant differences were found in pH values and soluble sugar content (Table 2). Regarding TPC, no significant differences were observed among treatments and the control, although RMtreated fruits showed a significant 54.7% increase in their in vitro antioxidant potential (Table 2). According to the data in Table 2, the percentages of N and C did

not show significant differences between herbicide and control samples. Furthermore, the phenolic profile of fruits was investigated by using an untargeted metabolomics approach based on UHPLC/QTOF mass spectrometry. Overall, 282 compounds could be annotated as polyphenols and used for further analysis (Supplementary Table S3). PLS-DA discriminant analysis was carried out to account for the differences in the phenolic pattern among the fruits from different treatments (Fig. 3). These discriminating polyphenols were classified according to their chemical class and subclass and included in Table 3. Among these compounds, flavonoids were the most represented phenolics, especially flavones and isoflavonoids. In addition, phenolic acids, such as hydroxycinnamic acids and phenolic terpenes, were represented. In agreement with the TPC assays, the RM and PA treatments induced a higher increase in phenolics, while MB caused a significantly lower phenolic content (particularly in terms of flavonoids).

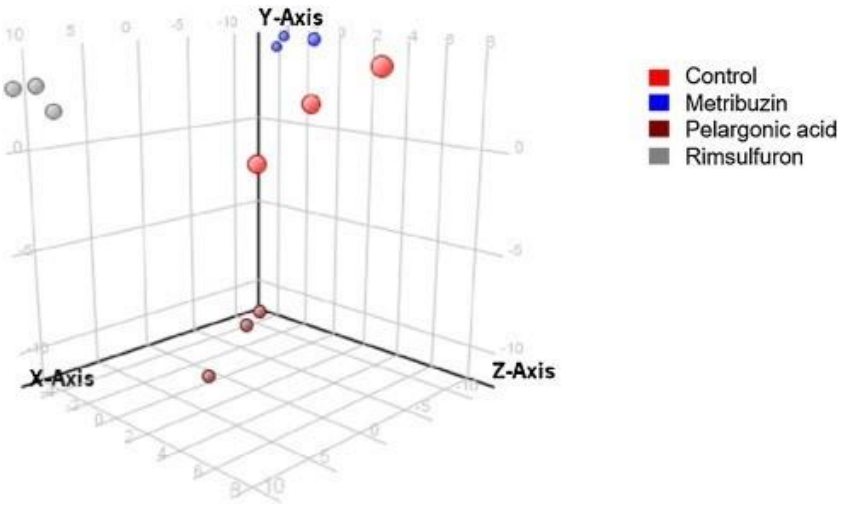


**Fig. 3. Partial least squares discriminant analysis (PLS-DA) score plot based on tomato fruit phenolic profiles obtained from UHPLC-ESI/QTOF-MS analysis following herbicide application.**

### 6.1.4 Discussion

Plants use a cascade of metabolic reactions for detoxifying xenobiotics, and most previous literature on this topic relates to pesticides. The process involves three distinct phases, namely activation, true detoxification, and excretion [21]. The earlier steps involve P-450 monooxygenases and peroxidases, followed by glutathione and glucosyl transferase conjugation,

cleavage reactions, and further Journal Pre-proof 13 conjugation to produce less toxic compounds [22], rather than transport and storage into the vacuole or cell wall binding [23]. However, there is growing evidence that the effects of pesticides go beyond the mere detoxification processes and may result in altered plant functions following treatments [4,6,24]. Pesticide application could result in morphological changes, such as changes in plant height or number of branches; alterations in membrane integrity; redox misbalancing; and accumulation of osmolytes [6,24–26]. These previous findings corroborate our results, which revealed that metabolomics profiles were altered by pesticide treatments even though the plants did not show any differences at the phenotype level (excepting for the non-selective herbicide Gly). The plants fresh weigh did not differ among treatments in the time from spraying to analysis (data not shown). Untargeted metabolomics allowed identifying profound biochemical reprogramming imposed by the treatments in tomato plants. Multivariate statistics based on the metabolomics signatures was used to describe similarities and dissimilarities across treatments. Interestingly, despite having a different mode of action, the cluster analysis revealed that similar metabolic profiles were presented in tomato plants after the application of the selective herbicides RM and MB. However, the tomato metabolome following PA and Gly treatment was more similar to the control but still distinctive. It has been proposed that herbicides may affect both primary and secondary metabolism [27], the latter playing an important role in adaptative plant responses to abiotic stress. Moreover, it has been proposed that herbicides such as EPSP and ALS inhibitors have a similar mode of action, involving carbon metabolism impairment [28]. In our study, RM and MB - which inhibit ALS and photosynthesis, respectively - had relatively more similar effects, while the EPSP inhibitor Gly clustered apart. Nonetheless, despite acting by foliar application, Gly treatment turned out to be lethal even when sprayed to the soil, and the plants prematurely died during the experiment. This indicates that plant metabolism was also affected by soil treatments, performed in our experiments in agreement with common agricultural practices. Journal Pre-proof 14 Concerning amino acids, a concurrent increase in glutamine could be observed, possibly indicating an effect on the GS-GOGAT nitrogen assimilation cycle. In this sense, RM has a direct impact on ALS activity and, because of that, on the biosynthetic rate of the precursors of leucine. However, several studies suggest that the effects of

MB on nitrogen metabolism are secondary since it may provoke nucleophilic displacement attacking protein synthesis and may determine the accumulation of ammonia and soluble N, whereas total N and protein contents are depressed [29]. In this regard, there is a link between the degradation of branched-chain amino acids and the biosynthesis of glucosinolates since Leu and Ile degradation products and Met biosynthesis intermediates are involved in the activation of this secondary pathway and, therefore, in plant defense [30,31]. Indeed, we observed a decrease in methionine and an increase in methionine-derived aliphatic glucosinolates. Glucosinolates play an important role in plant defense against herbivore attacks, but a growing body of literature reports their involvement in abiotic stress responses [32,33]. However, their physiological role is not fully elucidated, but several processes related to both plant morphology and physiology have been proposed [32,34]. Other secondary metabolites being altered by the treatments are the shikimatederived phenylpropanoids. A direct effect is expected in the case of Gly, which inhibits the synthesis of shikimate, whereas indirect effects are likely to occur in the case of other herbicides. Previous studies suggest that the phenylpropanoid pathway is not directly involved in the response to herbicides in the leaves of Solanum tuberosum L. since no differences were found by Zarzecka et al. [35] in the total phenolic content in plants treated with MB. Other studies found that flavonoids were not affected by herbicide treatments [36]. Interestingly, pesticides have been reported to induce oxidative stress in treated plants [37,38]. In fact, the observed increase in hydroxy- and other oxo-derivatives of fatty acids supports this point. Although phenolics play a pivotal role in plant defense against oxidative misbalances, in our experiments they were not recruited for this aim. Likely, other compounds, such Journal Pre-proof 15 as ascorbate and tocotrienols (both being modulated by the treatments), were acting to compensate for oxidative substances in the treated plants. Metabolomics also pointed out the involvement of isoprenoids in the plants' response to herbicides. Thus, plants treated with RM and MB presented a decrease in (R)-mevalonate-5phosphate, whereas presqualene diphosphate, a precursor of sterols and other triterpenes, decreased after RM application (Table 1). Furthermore, some sesquiterpenes were downregulated by RM and Gly. Although their role in the tomato plants' response to treatments is unclear, a pivotal role could be ascribed to another a class of terpenes showing

a strong upaccumulation, namely the gibberellins (GAs). These phytohormones are biosynthesized via the methylerythritol phosphate pathway (MEP) [39]; their biosynthetic intermediates were found to increase, whereas catabolites decreased. Notably, GAs are part of the whole multilevel hormone crosstalk that finally affects plant growth and responses to stimuli, acting antagonistically with cytokinins in leaf formation, epidermal differentiation, and meristem maintenance [40]. Interestingly, an increase in GAs has been correlated with increased stress tolerance. GA signaling involves DELLA proteins; emerging evidence indicates that this cascade is co-regulated by the stress hormone jasmonate [41]. Indeed, jasmonate precursors were strongly decreased in all herbicide-treated plants from our experiments. These hormones play a pivotal role in a plant's response to both abiotic and biotic stresses, mediating a series of growth processes in response to stimuli [42]. Therefore, metabolomics allowed depicting a wide metabolic reprogramming induced by the herbicides a few days after treatment. Surprisingly, this herbicide-related metabolic shaping occurring at the early growth stage could still result in distinct metabolic signatures in the fruits, i.e., at the reproductive stage of the plant. It is noteworthy that the time from treatment to harvest was rather large, being between 13 and 15 weeks, thus indicating that the effect of herbicides on metabolism was not transitory. Both productive and phytochemical changes could be observed in treated fruits compared to controls. Regarding productive parameters, the herbicide treatments did not significantly Journal Pre-proof 16 alter pH values, soluble sugar content, TPC, or N and C contents, with respect to untreated plants. In contrast, according to our results, MB and PA promoted a significant increase in tomato berries' fresh weight, whereas RM caused a significant increase in antioxidant activity. These results suggest a potential beneficial application of herbicides in that they could promote biomass production and antioxidant activity of the fruits. Concerning TPC, our findings are in line with those of Dragicevic et al. [43], who investigated the effect of six post-emergent herbicides on maize inbred lines. In contrast, other studies reported a significant reduction in polyphenolic content under herbicide treatments [35,44]. The effects of the herbicides used in this study, beyond the control of weeds, is also corroborated by the results of other authors, who reported a significant reduction in %C in herbicidetreated tomato berries compared to control plants [45]. However, in our study, the %C was not affected by any

treatment, likely because of compound-specific responses. This observation reinforces our hypothesis that, although herbicide application may not cause apparent phenotypic changes in tomato plants, the associated metabolic profile is markedly different. Interestingly, the effects of herbicides on fruit composition and yield suggest that it might be worthwhile to regard agronomic practices from a holistic perspective that also includes the quality of the products so that pesticide use can be correctly considered and planned.

### 6.1.5 Conclusions

An untargeted metabolomics approach was employed to investigate the hidden effects induced by selected herbicides on plant metabolism when used according to agronomic practices. Even when lacking phenotypic symptoms of toxicity or sufferance, the treated plants exhibited a largely modulated metabolic profile following the treatments. Although all tested herbicides presented different modes of action, some common responses in terms of metabolic profiles were found between MB- and RM-treated plants, while Gly- and PA-treated plants showed distinctive metabolic Journal Preproof 17 reprogramming and were more similar to the control plants. All the tested herbicides had the strongest effect on the phytohormone profile (mainly for gibberellins and jasmonates) and secondary metabolism, with several processes involved in the induced stress response. In this regard, phenylpropanoids, glucosinolates, and terpenes were altered. Surprisingly, such effects at the metabolic level were so relevant as to affect some qualitative and quantitative aspects of fruits, even though the herbicides were applied several weeks before. To date, these hidden effects have been largely underestimated even though nowadays it is difficult to imagine crop production without chemical inputs. However, the comprehension of these concealed aspects might provide important insights into the best strategies in crop defense, with the aim of improving food quality in addition to food safety. The present results provide both some positive and some negative aspects related to herbicide application. In turn, this means that the metabolic reshaping induced by herbicide use is not necessarily detrimental but, rather, is worth to be better understood and carefully considered in the framework of sustainable agricultural practices.

### 6.1.6 Supplementary materials

The tables and supplementary materials, listed below, is not presented in the text for reasons of space. Please refer to the following article:

Ganugi, P., Miras-Moreno, B., Garcia-Perez, P., Lucini, L., & Trevisan, M. (2020). Concealed metabolic reprogramming induced by different herbicides in tomato. *Plant Science*, 110727.

**Tab.1.** List of metabolites being differentially accumulated ($p < 0.05$, Bonferroni multiple testing correction; fold change > = 1.5) in tomato leaves' metabolomics profiles following treatment with the selected herbicides. Compounds are classified according to the PlantCyc Pathway Tool software and are provided together with individual log fold-change (FC) values, as compared to the control values.

**Tab.2**. Characterization of tomato fruits from plants treated with the selected herbicides. Fruits' fresh weight (average of the whole production per treatment), pH, sugar content, total phenolic compounds, antioxidant capacity (mg gallic acid equivalents per g), and nitrogen and carbon percentages are provided. Results are expressed as mean ± standard deviation (N = 3). Asterisks indicate significant differences compared to the control values ($p < 0.05$).

**Tab. 3.** Phenolic compounds being differentially accumulated ($p < 0.05$, Bonferroni multiple testing correction; fold change > = 2) in tomato berries following treatment with the selected herbicides. Compounds are classified into phenolic subclasses according to the Phenol-Explorer database annotations (www.phenol-explorer.eu). Log foldchange (FC) values are also provided.

**Tab. S1.** The whole dataset produced from untargeted metabolomics carried out in tomato leaves treated with herbicides. Compounds are presented with individual intensities and composite mass spectra.

**Tab. S2.** Differential metabolites in tomato leaf metabolomics profiles following herbicide application, as derived from Volcano Plot analysis ($p < 0.05$; fold change >= 1.5).

**Tab. S3.** The whole dataset of polyphenols annotated from untargeted metabolomics carried out in tomato fruits from plants treated with the selected

herbicides. Compounds are presented with individual intensities and composite mass spectra.

## 6.2 Evaluation of the Agronomic Traits of 80 Accessions of Proso Millet (*Panicum miliaceum* L.) under Mediterranean Pedoclimatic Conditions

### 6.2.1 Introduction

By 2050, the world's population is expected to have increased rapidly, from about 7 billion to 9.2 billion people, boosting the global food demand by up to 60% [46,47]. Currently, cereal crop consumption supports approximately 50% of the total calorie intake of the world and is largely supplied by wheat, rice, and maize [48-50]. At the same time, climate change is accelerating land degradation and desertification, and extreme climatic events are lowering yields [51-53]. Global warming may reduce arable land due to the expansion of dryland regions by around 10% by the end of the 21st century, increasing global food shortages and even famine, especially in developing countries where populations are already affected by malnutrition [54-56]. In view of the current and future scenarios, scientists suggest that an efficient strategy could be to replace high water-demanding cereal crops with drought-adopted ones, focusing on climate-resilient crops to ensure high productive and nutritional value by efficiently utilizing natural resources [57-59].

Among the $C_4$ Panicoids (subfamily: *panicoideae*), proso millet (*Panicum miliaceum* L.) is known to possess morpho-physiological traits, conferring tolerance to abiotic stresses and greater adaptability than major grain cereal crops under different environmental conditions [60-61]. Based on the panicle morphology and shape, proso millet can be divided into five races: *miliaceum*, *patentissimum*, *contractum*, *compactum*, and *ovatum* [62-63]. *Panicum miliaceum* is one of the first domesticated crops in the world, and it was cultivated before the diffusion of rice, maize, and wheat [64-65]. Ten thousand years ago, it appeared as a staple food in the semiarid regions of East Asia (e.g., China, Korea, Japan, Russia, and India), and later spread throughout the entire Eurasian region [66-67]. Nowadays, millet grains still represent an important cereal food as a source of energy and protein for

millions of people living in arid and semiarid areas in emerging countries, while millet biomass represents an interesting source of forage in some Asian countries, such as India [68-69]. In the Western world, *P. miliaceum* is considered a minor cereal due to its poor economic importance, and thus, it is usually used as feed or fodder for farm animals [64,66].

Compared to the other cereals, proso millet may represent a valuable crop, especially in Mediterranean areas, for its nitrogen use efficiency (NUE)—which is 1.5–4 times greater than that of $C_3$ cereals—its high leaf area index (LAI; 6.7), and its high radiation use efficiency (RUE; 2.5–4 g $MJ^{1}$), which are comparable with that of maize growth under optimal conditions [67,70]. To produce 1 g of biomass, it requires about half the water that is needed to produce an equal amount of maize or wheat biomass [53,71]. *Panicum miliaceum* has recently received increasing interest due to its nutraceutical traits: grains are characterized by a high protein content (12.5%) and are generally rich in essential amino acids (e.g., methionine and cysteine), with the exception of lysine and threonine [57,65]. Human foods containing millet are promoted for their low glycemic index and their high fiber content, as well as for being gluten-free [72,73]. All of the above makes millet a potential candidate to source stress tolerance and nutritional traits for next-generation cereal breeding programs [60].

The objective of this study was to conduct an overall morphological characterization and a preliminary evaluation of the agronomical performances of a world collection of 80 proso millet (*P. miliaceum*) accessions over a 2 year field experiment. The collection used to estimate the agronomic trait values had several geographical proveniences and showed a broad variation for a number of traits. The results represent a useful tool for designing and implementing breeding programs aiming at the production of new and improved varieties.

### 6.2.2 Materials and methods

*Field Experiments*

From a germplasm collection of wild and domesticated proso millet accessions (nearly 600 accessions) sourced from the United States Department of Agriculture, Darby, PA, USA [74], we selected 80 accessions

to use in this study. The selection was done according to the results (data not shown) of a preliminary agronomical field screening carried out in 2017. The selected accessions showed the best promising traits and interesting seed colors, and possessed a complete bank passport of information (origin, senders, etc.). Our collection featured cultivated materials with several different countries of origin: Central Asia (n = 1), South Asia (n = 7), Southeast Asia (n = 8), West Asia (n = 15), East Asia (n = 3), North America (n = 1), Central America (n = 8), South America (n = 2), North Africa (n = 4), East Africa (n = 1), Central Europe (n = 7), Western Europe (n = 6), Eastern Europe (n = 13), and Oceania (n = 4). A detailed list of the materials with the corresponding passport information is provided in Supplementary Table S1.

The field evaluation of the collection was conducted for two consecutive years (i.e., 2018 and 2019) at the experimental farm belonging to the Tuscan Regional Administration located in Cesa (AR), Italy (43°18 32.4 N; 11°49 35.1 E; 253 m a.s.l). At the farm, the climate is typically Mediterranean and characterized by an average yearly temperature of 13.9 , with a minimum average temperature of 5.8 °C in January and a maximum average temperature of 24.0 °C in July. Typically, the annual precipitation ranges from 685 to 711 mm distributed across 89 rainy days (i.e., with rainfall above 1 mm). The meteorological data during the growing experimental period were recorded at the local weather station (Table 1). The experimental soil was characterized by a clay texture (25.4% sand; 30.1% silt; 44.5% clay), a pH of 7.1, low electrical conductivity (EC; 0.154 mS cm 1), high cation exchange capacity (CEC; 27.46 meq 100 g 1), and an organic matter content of 1.66% [27 72] (Table 2).

**Tab. 1.** Meteorological data for the experimental site during agronomic seasons 2018 and 2019.

| Month | Mean Temperature (°C) | | Mean T Max (°C) | | Mean T Min (°C) | | Total Rainfall (mm) | | Mean Relative Humidity (%) | |
|---|---|---|---|---|---|---|---|---|---|---|
| Year | 2018 | 2019 | 2018 | 2019 | | | 2018 | 2019 | 2018 | 2019 |
| | | | | | 12.0 | 8.0 | | | | |
| January | 6.7 | 2.5 | | | | | 27.8 | 38.8 | 87.2 | 83.5 |
| February | 3.9 | 6.8 | 8.2 | 14.4 | | 0.0 | 93.8 | 39.6 | 81.3 | 68.7 |
| | | | | | | 2.1 .4 | | | | |
| March | 7.8 | 9.8 | 12.8 | 17.2 | 2.9 | | 127.2 | 5,2 | 81.7 | 64.7 |
| April | 14.6 | 11.9 | 21.6 | 18.5 | 6.9 | 5.2 | 66.4 | 98.0 | 70.6 | 73.9 |
| | | | | | 11.6 | 8.2 | | | | |
| May * | 17.6 | 13.5 | 23.6 | 18.9 | | | 140.6 | 128.6 | 77.2 | 80.1 |
| June * | 20.4 | 22.3 | 27.7 | 30.7 | 13.1 | 13.8 | 14.0 | 2.2 | 65.3 | 61.5 |
| | | | | | 15.4 | 15.8 | | | | |
| July * | 23.7 | 24.0 | 32.0 | 32.2 | | | 22.6 | 202.6 | 61.3 | 62.7 |
| August * | 23.9 | 24.4 | 32.1 | 32.5 | 15.1 | 16.2 | 23.0 | 45.4 | 62.7 | 66.3 |
| | | | | | 12.8 | 12.1 | | | | |
| September | 20.2 | 19.4 | 28.2 | 26.7 | | | 18.6 | 34.2 | 66.9 | 72.9 |
| October | 15.9 | 15.2 | 22.3 | 22.2 | | 9.3 | 59.2 | 59.2 | 74.1 | 84.4 |
| | | | | | 9.8 | 7.1 | | | | |
| November | 10.2 | 11.0 | 14.3 | 15.2 | 6.6 | | 102.4 | 207.0 | 89.0 | 91.0 |

| Month | Mean Temperature (°C) | | Mean T Max (°C) | | Mean T Min (°C) | | Total Rainfall (mm) | | Mean Relative Humidity (%) | |
|---|---|---|---|---|---|---|---|---|---|---|
| Year | 2018 | 2019 | 2018 | 2019 | | | 2018 | 2019 | 2018 | 2019 |
| | | | | | 10.2 | 12.4 | | | | |
| December | 5.1 | 7.1 | | | | | 63.8 | 90.0 | 90.8 | 86.9 |
| **Mean** [a] | **21.4** | **21.1** | **28.8** | **28.6** | **13.8** | **13.5** | **50.1** | **94.7** | **66.6** | **67.7** |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Total Rain** [b] | - | - | - | - | - | - | **200.2** | **378.8** | - | - |
| **Total Rain** [c] | - | - | - | - | - | - | **759.4** | **950.8** | - | - |

a The monthly mean for the whole growth period, from planting to maturation; b The cumulative rainfall during grain development; c The cumulative year rainfall; * Months in which the crop was in the field.

**Tab. 2.** Physical and chemical properties of the soil used in the experiment.

| Properties | Value | Unit |
|---|---|---|
| Sand | 25.4 | % |
| Silt | 30.1 | % |
| Clay | 44.5 | % |
| Total organic matter | 1.66 | % |
| Total nitrogen | 0.12 | % |
| Available phosphorous | 11 | ppm |
| pH | 7.1 | |
| Electrical conductivity (EC) | 0.154 | mS $cm_1$ |
| Cation exchange capacity (CEC) | 27.46 | meq 100 $g_1$ |
| Exchangeable Ca | 21.25 | ppm |
| Exchangeable Mg | 5.17 | ppm |
| Exchangeable Na | 0.58 | ppm |
| Exchangeable K | 0.46 | ppm |

During both years, the experimental layout was of a randomized complete block design with two replications for each accession (total of 160 plots). The planting density was 55 plants m 2, with a distance between rows of 60 cm and 3 cm between the plants in each row. The applied planting density was adopted based on recent studies [75] in order to optimize and standardize the experiment using an average density used in open field. Each experimental plot measured 2.4 m in length and 3 m in width (7.2 m2), and was sown, as

classically happen for spring crops in that area, at the beginning of May (4 May 2018 and 9 May 2019). Harvesting was performed on 25 August 2018 and on 30 August 2019. The precedent crop in both years was bread wheat (*Triticum aestivum*). The seedbed was prepared using a disc harrow (20–25 cm depth) in winter, followed by a spike-tooth harrow (6–8 cm depth) before seeding. Fertilization was applied at sowing time using 150 kg ha 1 of NPK (10-10-10), the adoption of a limited supply of fertilizers is motivated by the desire to minimize the influence of this factor on the experimental device since the aim was precisely to verify the vegetative-productive behavior of the accessions in standard conditions. The following crop management was performed using manual weeding and without the use of irrigation and pesticides, in fact weeds generally represent a big problem for the cultivation of millet but in our case, given the experimental nature of the cultivation and the limited degree of infestation, we proceeded to perform two manual weeding. Regarding irrigation, since the purpose of the experiment was to determine the feasibility of the cultivation of millet in semi-arid areas and with reduced water consumption, together with the satisfactory rainfall recorded in the two years of activity, it was considered important do not administer water by irrigation. Finally, both thanks to the careful agronomic management of the field trial, and thanks to a favorable climatic course during the two years of experimentation, no parasitic attacks such as to require phytosanitary treatments have been carried out.

*Data Collection*

Harvesting was performed when the seeds were fully matured according to the phenological BBCH (Biologische Bundesanstalt, Bundessortenamt, und Chemische Industrie) scale [76]. At maturity, the plants were classified in different P. miliaceum races according to their panicle morphology and shape [62,63]. Three randomly selected plants for each plot were harvested and used to measure the vegetative traits, such as plant height, leaf number, basal tiller number, and grain yield per plant. Plant height was measured as the distance from the ground level to the end of panicle. For each experimental plot, a 0.50 m2 area was harvested by hand, oven-dried at 55 °C for 48 h, and then used to estimate the productive traits (i.e., total dry biomass, grain yield, and 100-seed weight). Total dry biomass and grain yield were subsequently

converted into kilograms per hectare (kg ha 1). The harvest index (HI) was calculated as the ratio between grain yield and total dry biomass of the whole sampled plants. This parameter indicates the partitioning of photosynthesis products to the harvestable portion of the crop [77]. Days to maturity were calculated from the emergence stage to the harvest cultivation stage, while cumulative Growing Degree Days (GDDs) were recorded from the emergence until at least 50% of the plants in a plot had reached the flowering phenological stage or beyond, using the following formula [78]:

GDD = ∑i=1n DTT

(1)

DTT = [(Tmax + Tmin)/2]  Tbase

(2) where DTT is the daily thermal time recorded, Tmin is the minimum daily temperature (°C), Tmax is the maximum daily temperature (°C), Tbase is the base air row temperature set equal to 10 °C for millets and sorghum, and i = 1,2,3,4…n are the days for which cumulative GDDs is calculated [79,80]. In addition, considering the importance of GDDs in proso millet growth and productivity, the accessions were subdivided into the following precocity classes: early, medium, and late maturity [77,81,82].

*Statistical Analysis*

The differences in the agronomic performances among accessions were assessed using a general linear mixed model (GLMM), fitted using R software v3.6 with R/lme4 package [84,85], and considering years and accessions as the random factors and the assigned morphological race as the fixed factor. A post-hoc Tukey test for multiple comparisons among morphological races was carried out using the same software. The correlation between all of the collected variables was assessed using a Pearson correlation model implemented in the R/corrplot package [85]. A principal component analysis (PCA) on the vegetative and production traits was carried out considering the overall mean values of both years using the R/factoextra [85], in order to estimate the relative importance of each trait in capturing data variation and the importance of the morphological race, origin, and GDDs to flowering parameters as possible factors structuring the germplasm collection. In addition, a non-parametric multivariate analysis of variance (np-MANOVA) was performed to test the differences among the clusters previously

hypothesized by using R/vegan [83]. Finally, the broadsense heritability (h2b) was estimated for each trait in each experimental year according to Equation (3) and using the software METAR v6.04 (CIMMYT Research Data & Software Repository Network) [86], and classified as low (<0.30), moderate (0.30–0.60), or high (>0.60) according to Vetriventhan and Upadhyaya [87].

The broad-sense heritability of a given trait at an individual environment is calculated as:

h2b = 2g/ 2g + ( 2e/nreps)

where 2g and 2e are the genotype and error variance components, respectively, and nreps is the number of replicates.

### 6.2.3 Results

*Variability for Agronomic Traits and Heritability*

The 80 accessions of proso millet could be classified into four races: miliaceum (52.5%), contractum (22.5%), patentissimum (17.5%), and compactum (7.5%). No accession in the collection was identified as being from the ovatum race. The outcomes of the GLMM model indicate that the genotypes differed significantly ($p < 0.01$) for plant height, grain yield, total dry biomass, HI, GDDs to flowering, and days to maturity among the single years (Table 3). All proso millet accessions showed a wide range of variability for all of the measured traits, especially for plant height (25– 104 cm in 2018 and 33–111 cm in 2019), grain yield (842–2982 kg ha 1 in 2018 and 891–3125 kg ha 1 in 2019), total dry biomass (2889–9664 kg ha 1 in 2018 and 2767–10,627 kg ha 1 in 2019), HI (0.25–0.33 in 2018 and 0.27– 0.35 in 2019), GDDs to flowering (581–891 in 2018 and 592–899 in 2019), and days to maturity (80–109 days in 2018 and 83–111 days in 2019) for both years. Overall, the accessions showed significantly ($p < 0.01$) lower mean values in 2018 compared to 2019 for plant height (67.48 cm and 69.82 cm, respectively), grain yield (1708 kg ha 1 and 1832 kg ha 1, respectively), total dry biomass (6001 kg ha 1 and 6279 kg ha 1, respectively), HI (0.28 and 0.30,

respectively), GDDs to flowering (740.8 and 743.3, respectively), and days to maturity (97.8 days and 98.8 days, respectively) (Table 3).

Plant height, leaf number, seed yield per plant, grain yield, total dry biomass, GDDs to flowering, and days to maturity were found to significantly differ between races ($p < 0.01$ for all parameters) (Table 4). The compactum race was characterized by a short plant (53.6 cm) and the lowest leaf number (5.99 on average), and produced the lowest grain yield and total dry biomass (1428 kg ha 1 and 4902 kg ha 1, respectively). Plants of the compactum type required less GDDs than the other races to reach the flowering stage (679 GDDs) and were characterized by early maturity (86 days). On the contrary, the accessions of contractum and miliaceum produced the highest average grain yield (1900 kg ha 1 and 1860 kg ha 1, respectively), total dry biomass (6506 kg ha 1 and 6432 kg ha 1, respectively), and plant height (75.1 cm and 71.5 cm, respectively), but differed for leaf number (7.11 and 6.56, respectively), seed yield per plant (10.28 g and 8.94 g, respectively), GDDs to flowering (772 and 732, respectively), and days to maturity (98 days and 95 days, respectively). The race patentissimum exhibited intermediate values for plant height (69 cm), grain yield (1790 kg ha 1), total dry biomass (6291 kg ha 1), and days to maturity (92 d), yet did not differ significantly from the race miliaceum for leaf number, seed yield per plant, and GDDs to flowering.

In general, the estimates of broad-sense heritability were found to be moderate–high for all of the agronomic traits evaluated, ranging from 0.53 for total dry biomass to 0.85 for plant height in 2018 and from 0.58 for grain yield and total dry biomass to 0.87 for leaf number in 2019 (Table 3). High heritability (>0.60) was observed in both years for plant height (0.85– 0.86), leaf number (0.82–0.87), basal tiller (0.82–0.83), seed yield per plant (0.71–0.75), 100-seed weight (0.73–0.77), GDDs to flowering (0.77– 0.79), and days to maturity (0.73–0.75). The millet collection exhibited moderate heritability for grain yield (0.55–0.58), total dry biomass (0.53–0.58) and HI (0.58–0.59).

**Tab. 3.** Mean, range, and broad-sense heritability ($h^2_b$) of the agronomic traits evaluated in the two-year field experiment.

| Trait | 2018 | | | 2019 | | |
|---|---|---|---|---|---|---|
| | Mean | Range | $h^2_b$ | Mean | Range | $h^2_b$ |
| Plant height (cm) | 67.48 b | 25–104 | 85 | 69.82 a | 33–111 | 0.86 |
| Leaf number | 6.70 a | 3–11 | 0.82 | 6.52 a | 3–10 | 0.87 |
| Basal tiller | 3.9 a | 2–6 | 83 | 3.7 a | 2–6 | 0.82 |
| Seed yield per plant (g) | 8.54 a | 2.6–16.7 | 0.71 | 8.96 a | 2.8–15.9 | 0.75 |
| Grain yield (kg $ha_1$) | 1708 b | 842–2982 | 55 | 1832 a | 891–3125 | 0.58 |
| Total dry biomass (kg $ha_1$) | 6001 b | 2889–9664 | 0.53 | 6279 a | 2767–10,627 | 0.58 |
| Harvest index | 0.28 b | 0.25–0.33 | 58 | 0.30 a | 0.27–0.35 | 0.59 |
| 100-seed weight (g) | 0.56 a | 0.35–0.71 | 0.73 | 0.54 a | 0.32–0.71 | 0.77 |
| GDDs to flowering | 740.8 b | 581–891 | 77 | 743.3 a | 592–899 | 0.79 |
| Days to maturity | 97.8 b | 80–109 | 0.73 | 98.8 a | 83–111 | 0.75 |

Means followed by the same letters in the same row are not significant at $p > 0.05$, while means followed by different letters in the same row are significant at $p < 0.05$. GDDs, growing degree days.

**Table 4.** Mean and range of the agronomic traits evaluated in proso millet races based on the mean data of the two years.

| Trait | Mean | | | | Range | | | |
|---|---|---|---|---|---|---|---|---|
| | ***compactum*** | ***contractum*** | ***miliaceum*** | ***patentissimum*** | ***compactum*** | ***contractum*** | ***miliaceum*** | ***patentissimum*** |
| Plant height (cm) | 53.6 c | 75.14 a | 71.52 ab | 68.8 b | 34–62 | 53–97 | 38–100 | 58–90 |
| Leaf number | 5.99 c | 7.11 a | 6.56 b | 6.18 b | 4–7 | 5–10 | 4–9 | 4–9 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Basal tiller | 3.6 a | 3.9 a | 3.9 a | 3.9 a | 3–5 | 3–5 | 3–5 | 4–5 |
| Seed yield per plant (g) | 6.64 c | 10.28 a | 8.94 b | 8.22 b | 3.7–12.1 | 6.1–19.1 | 4.6–14.1 | 3.7–13.8 |
| Grain yield (kg ha$_1$) | 1428 b | 1900 a | 1860 a | 1790 ab | 911–2045 | 1209–2984 | 994–2893 | 1004–2743 |
| Total dry biomas s (kg ha$_1$) | 4902 c | 6506 a | 6432 a | 6291 b | 2909–7550 | 3960–9775 | 3422–9477 | 3334–9230 |
| Harves t index | 0.28 a | 0.30 a | 0.30 a | 0.29 a | 0.25–0.31 | 0.26–0.34 | 0.26–0.35 | 0.26–0.33 |
| **Trait** | **Mean** | | | | **Range** | | | |
| | ***compact um*** | ***contract um*** | ***niliace um*** | ***patentissim um*** | ***compact um*** | ***contract um*** | ***miliace um*** | ***patentissim um*** |
| 100seed weight (g) | 0.54 a | 0.55 a | 0.55 a | 0.54 a | 0.36–0.69 | 0.45–0.69 | 0.46–0.68 | 0.43–0.67 |
| GDDs to floweri ng | 679 c | 772 a | 732 b | 721 b | 590–760 | 609–890 | 606–886 | 602–881 |
| Days to maturi ty | 86 b | 98 a | 95 b | 92 ab | 83–91 | 92–111 | 90–108 | 88–105 |

Means followed by the same letters in the same row are not significant at $p > 0.05$, while means followed by different letters in the same row are significant at $p < 0.05$.

The first three principal components (PCs) computed for the agronomic traits explained about 71% of the total variation among the traits evaluated (Figure 1 and Table 5). GDDs to flowering, grain yield, total dry biomass, days to maturity, and plant height were the most important variables, and contributed largely to the first principal component (PC1; 0.623, 0.582, 0.533, 0.449, and 0.425, respectively), explaining 33% of the total variation. The second component (PC2) accounted for 25% of the total variation and differentiated the accessions by seed yield per plant and days to maturity (0.536 and 0.374, respectively). The third component (PC3) explained an additional 13% of the total variation and was attributed 100-seed weight for positive loadings (0.513) and basal tillers for negative loadings (0.783). In the np-MANOVA results, the origin and race membership did not seem to represent good clustering factors ($p > 0.05$ for both), but three major groups were identified according to the cumulative GDDs to flowering groups ($p < 0.05$). The group in the top area of the PCA graph (Figure 1), named A, contained the early-flowering proso millet accessions (590–690 GDDs); the lower group, named C, comprised the late-flowering accessions (790–890 GDDs); and the central group, named B, contained the mediumflowering accessions (690–790 GDDs).

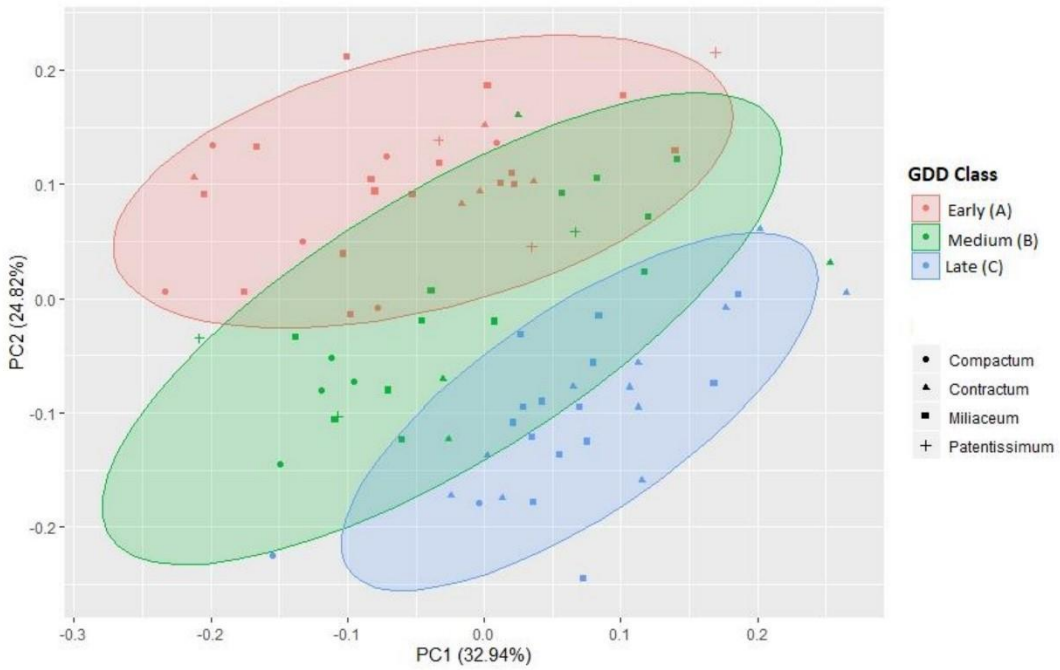


**Fig. 1**. Principal component graph of vegetative and productive traits based on the first two components.

Colors represent the three different GDD clusters while point form the race of each accession. Group A = Early flowering accessions (560–690 GDD); Group B = Medium flowering accessions (690–790 GDD); Group C = Late flowering accessions (790–890 GDD).

The correlation coefficients between the agronomic traits are reported in Table 6. Grain yield showed a high correlation (r = 0.688) with total dry biomass, and both reported intermediate correlations with plant height (r = 0.445 and r = 0.436, respectively), seed yield per plant (r = 0.538 and r = 0.521, respectively), GDDs to flowering (r = 0.680 and r = 0.594, respectively), and days to maturity (r = 0.655 and r = 0.638, respectively), and low correlations with leaf number (r = 0.288 and r = 0.287, respectively). GDDs to flowering had a positive significant correlation with days to maturity (r = 0.741), and the same positive pattern was also observed between plant height and leaf number, GDDs to flowering, days to maturity, and seed yield plant (r = 0.547, r = 0.300, r = 0.309, and r = 0.244, respectively).

**Table 5.** Principal component analysis factor loadings (PC1, PC2, and PC3) among the agronomic traits in proso millet based on the mean data of the two years.

| Trait | PC1 | PC2 | PC3 |
|---|---|---|---|
| Plant height (cm) | 0.425 | □0.074 | □0.264 |
| Leaf number | 0.351 | 0.207 | □0.234 |
| Basal tiller | □0.079 | 0.112 | □0.783 |
| Seed yield per plant (g) | 0.309 | 0.536 | □0.034 |
| Grain yield (kg ha□¹) | 0.582 | 0.198 | 0.097 |
| Total dry biomass (kg ha□¹) | 0.533 | 0.234 | 0.136 |
| 100-seed weight (g) | □0.053 | 0.213 | 0.513 |
| GDDs to flowering | 0.623 | 0.189 | 0.097 |
| Days to maturity | 0.449 | 0.374 | 0.023 |
| Standard deviation | 1.9217 | 1.4947 | 1.0778 |
| Proportion of variance | 32.941 | 24.823 | 12.947 |
| Cumulative proportion | 32.941 | 57.764 | 70.711 |

**Table 6.** Correlation coefficients among the agronomic traits in proso millet based on mean data of two years.

| Trait | Plant Height (cm) | Leaf Number | Basal Tiller | Seed Yield Per Plant (g) | Grain Yield (kg ha$_1$) | Total Dry Biomass (kg ha$_1$) | Harvest Index | Days to Maturity | 100Seed Weight | GDDs to Flowering |
|---|---|---|---|---|---|---|---|---|---|---|
| Plant height (cm) | 1 | - | - | - | - | - | - | - | - | - |
| Leaf number | 0.547 ** | 1 | - | - | - | - | - | - | - | - |
| Basal tiller | 0.062 | 0.007 | 1 | - | - | - | - | - | - | - |
| Seed yield per plant (g) | 0.244 * | 0.167 | 0.094 | 1 | - | - | - | - | - | - |
| Grain yield (kg ha$_1$) | 0.445 ** | 0.288 ** | 0.225 * | 0.538 ** | 1 | - | - | - | - | - |
| Total dry biomass (kg ha$_1$) | 0.436 ** | 0.287 ** | 0.485 * | 0.521 ** | 0.688 ** | 1 | - | - | - | - |
| Harvest | 0.08 | 0.115 | 0.12 | 0.09 | 0.11 | 0.182 | 1 | - | - | - |
| **Trait** | **Plant Height (cm)** | **Leaf Number** | **Basal Tiller** | **Seed Yield Per Plant (g)** | **Grain Yield (kg ha$_1$)** | **Total Dry Biomass (kg ha$_1$)** | **Harvest Index** | **Days to Maturity** | **100Seed Weight** | **GDDs to Flowering** |
| Index | 6 | | 1 | 5 | 8 | | | | | |
| Days to maturity | 0.309 ** | 0.259 * | 0.176 | 0.100 | 0.655 ** | 0.638 ** | 0.125 | 1 | - | - |
| 100seed weight (g) | 0.147 | 0.155 | 0.223 | 0.143 | 0.052 | 0.073 | 0.027 | 0.206 | 1 | - |
| GDDs to flowering | 0.300 ** | 0.255 * | 0.175 | 0.108 | 0.680 ** | 0.594 ** | 0.125 | 0.741 ** | 0.206 | 1 |

* Significant at $p < 0.05$; ** Significant at $p < 0.01$.

### 6.2.4 Discussion

In this study, we evaluated the agronomic performances of 80 different proso millet accessions in two consecutive cultivation years (2018 and 2019) under Mediterranean pedoclimatic conditions. The characterization of the agronomic traits of germplasms is considered an important step to select genotypes adaptable to different environments and with desirable productive traits to be used in future breeding programs for the constitution of new improved varieties [88].

Overall, the field experiment of 2019 showed better agronomic traits values than that of 2018. This could be attributed to the drought stress that occurred during the ear emergence stage, which negatively affected plant growth [59] in the first experimental year (i.e., 2018). The morphological and productive variability of the P. miliaceum accessions and races assessed during the 2 year field experiment was comparable to that of other studies on millet germplasm evaluation [61,78,87,89]. For example, plant height ranges recorded were found to be within the range from 39 to 173 cm reported by Salini [90], also consistent with the other small millets species which exhibited similar values, such as pearl milled (62–160 cm) and barnyard millet (79–156 cm), with the only exception being kodo millet, which exhibits shorter plants (34–101 cm) [87-89,91] Moreover, our data confirmed that the compactum race was characterized by short and low grain yielding plants compared to the contractum, miliaceum, and patentissimum races [61,90] suggesting that is unlikely that all accessions of the compactum race evaluated are suitable for mechanical harvesting due to grain losses during cutting operations [92].

In general, worldwide cultivation of proso millet has declined in the last decade, particularly because of its low grain yield (average world yield of 890 kg ha 1) compared to the major cereal crops [47,65]. Although the response of the grain yield parameter does not depend only by the genotype chosen, but also on the rainfall, temperatures, and agricultural techniques applied [71,77], the present results showed that high-yielding accessions could reach 3125 kg ha 1 of cereal grain. This is in agreement with previous studies that reported that the use of a promising genotype leads to an average grain yield of 2016 kg ha 1 in the United States and up to 2600 kg ha 1 in India [61,93]. In addition, the correlation results indicated that the performances of the accessions were positively correlated with plant height and leaf number,

probably because these traits are linked to intercepting more light, thereby increasing photosynthesis efficiency [93,94], it is however important to keep in mind that some pearl millet genotypes have been breed to limit height in very dry area to avoid lodging. Indeed, millets are known to accumulate more dry matter compared to wheat, maize, and sorghum, producing biomass useful for providing forage and biogas production [77,95,96]. In fact, this parameter can reach 13,961 kg ha 1 under dryland conditions and 14,407 kg ha 1 when irrigated in a short period of time [97].

Crops characterized by low grain yield and high total biomass weight generally result in low HI ratios [77,98]. Among these, P. miliaceum and other millets (i.e., Pennisetum glaucum, Setaria italica, and Echinochloa frumentacea) have been reported to have lower HI ratios (i.e., 0.25–0.35, 0.20–0.30, 0.30–0.35, and 0.36–0.41, respectively) compared to major cereal crops (0.40–0.60) such as wheat, rice, maize, and sorghum [77,99101]. The rise in HI ratio also results in an increase in grain yield, and thus might be an important goal in breeding programs. This could be achieved by reducing tillering, as occurs in maize and sorghum, because intra-plant competition for assimilates between tillers and seeds is considered a major cause of low grain yield [94,98]. The low effect of basal tiller on grain yield was previously noted in barnyard and proso millet, suggesting that this trait is less relevant and that its improvement could be of low priority [89,90]. Millets have a relatively short maturation time (3–4 months), which makes it a desirable crop for sustainable rain-fed agriculture [71]. Generally, among millet groups, proso requires less GDDs than foxtail and pearl millet to reach the flowering stage [102-104]. However, the entire P. miliaceum germplasm tested covered a very large range of precocity, as previously recorded by Vetriventhan and Upadhyaya [61] based on the ICRISAT (International Crops Research Institute for the Semi-Arid Tropics, Patancheru, India) germplasm, by Sanon et al. [76] based on the local millet varieties cultivated in West Africa, and by Salini et al. [90] based on 364 accessions tested at the Department of Millets, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatroe, India. From the results of the non-parametric multivariate analysis of our data, it was possible to identify three classes of precocity based on cumulative GDDs to flowering. The late-flowering millet accessions required high cumulative GDDs and resulted in a better yield production compared to the early and medium ones, due to the

longest growing cycle, which increased the grain yield and biomass accumulation [88,89,105]. On the contrary, Eric et al. [106] reported the lowest grain yield using late maturity accessions, because the plants flowered under limited moisture conditions when the amount of rainfall was less than at the beginning of the season. These findings suggest that breeding programs should try to develop new and improved varieties within the different precocity classes suitable for different environmental conditions. Moreover, our data do not show any evidence of accession clusterization according to their geographical origin, in contrast to what was found by Rajput and Santra [107]. In fact, considering our data, the accessions share similar trait combinations independently by their origin or their race. This could be due to the short history of breeding this crop or the heterogeneous group selected in each area [108]. Therefore, the passport information obtained from the germplasm bank could contain inaccurate attributions concerning the taxonomy identification made by the seed donors.

At the same time, the heritability estimates of the single traits suggest that the collection studied could be appropriate for effective selection [109]. In fact, according to several authors, plant height, leaf number, basal tiller, seed yield plant, 100-seed weight, GDDs to flowering, and days to maturity in millets underline a high genetic component ($h^2_b > 0.7$) and represent good traits to be selected in a breeding program [87,88,90]. On the contrary, the selection of traits with moderate heritability, such as grain yield, total dry biomass, and HI, would be difficult to set up. This could be achieved by indirect selection using the high heritability traits characterized by a high positive correlation with the trait to be improved [89,90,106]. Finally we should state that despite the promising yields and the positive traits that may confer resilience to environmental stress, P. miliaceum remains a minor cereal primarily cultivated in semi-arid, low input dryland agriculture regions of Africa and southeast Asia, as a subsistence crop for local consumption, with very limited quantities recorded in international trade of West Countries [64]. Commercial millet production is risky for Western farmers, because the absence of large market outlets means that fluctuations in output cause significant price fluctuations [66]. Many specific agronomic constraints affect its cultivation (poor soil fertility, low and erratic rainfall, high temperature after sowing, loss of grain to birds, pest and weed management) limiting its spread and forcing to develop new technologies for crop and resource

management, in order to promote its production on a large scale, including breeding of new varieties [110]. Moreover, only limited experience has been acquired on millet breeding in developed countries, compared what has been done for wheat and maize [111]. Among the different types of millet only pearl millet, and to a small extent finger millet, has so far been researched at the international level. Hybrid grain cultivars have been developed for pearl millet in India and the United States, but perform best in areas where rainfall is reliable [112]. In drier areas with limited and fluctuating rainfall, where it is difficult for breeders to identify dual-purpose grain/stover modern varieties, openpollinated varieties or composite cross population (CCP) that give stable grain and straw yields and suit the prevailing rainfall pattern, should be developed and adopted [56,113].

### 6.2.5 Conclusions

Despite the decline in P. miliaceum cultivation due to its low grain yield, this crop has generated great interest in recent years as a promising sustainable candidate for the Tuscanian semi-arid zones as a renewal crops in a sustainable rotational crop program, which could contribute to crop diversification, as well as diet and use of land. Its planting time, indeed, fits well in rotation with winter annual crops such as winter wheat or warmseason broadleaf crops such as sunflower or sugar beet. In this study, the evaluation of the agronomical performances of 80 accessions over a 2 year field experiment showed that the entire collection under evaluation exhibited a wide range of variability for plant height, grain yield, total dry biomass, HI, GDDs to flowering, and days to maturity. Overall, the I.Pm. 673 (ID: Ames 11678; India) and GR 665 (ID: PI 517019; Morocco) accessions showed the highest grain yield, while GR 658 (ID: PI 517017; Morocco) and Index Seminum #568 (ID: PI 649371; Germany) reported a greater total dry biomass. All of this information could be utilized in future breeding programs for the development of new and improved genotypes adaptable to different environments and with desirable productive traits.

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