Doctoral (PhD) dissertation

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Improvement of Winter Wheat (*Triticum aestivum* L.) Drought Tolerance via Biotechnology-Generated Genotypes

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LIST OF ABBREVIATIONS

1- Text

2, 4-D	2,4-Dichlorophenoxyacetic acid
QTL	Quantitative trait locus
ZEN	Zearalenon
APM	Aminoprophos-methyl
STI	Stress tolerance index

2- Tables and Figures

AGB	Above-ground biomass
AGB.R	Above-ground biomass reduction
ANOVA	Analysis of variance
CV	Coefficient of variation
DF	Degrees of freedom
DS	Drought stress
FSN/p	Fertile spikelet number/plant
FSN/p.R	Fertile spikelet number/plant reduction
GN/p	Grain number/plant
GN/p.R	Grain number/plant reduction
GY/p	Grain yield/plant
GY/p.R	Grain yield/plant reduction
HI	Harvest index
HI.R	Harvest index reduction
HT	Heading time
LSD	Least significant difference
MS	Mean square
MSL	Main spike length
MSL.R	Main spike length reduction
PH	Plant height
PH.R	Plant height reduction
Pr	Probability
RDM	Root dry mass
RDM.R	Root dry mass reduction
RL	Root length
SD	Standard deviation of the mean
SE	Standard error of the mean
SPN/p	Spikelet number/plant
SPN/p.R	Spikelet number/plant reduction
SS	Sum of squares
STI	Stress tolerance index
TGW	1000-grain weight
WW	Well-watered

1. INTRODUCTION

Common wheat (*Triticum aestivum* L.) is one of the main strategic cereal crops in the world, grows in different environments, from temperate, irrigated to dry and high-rain-fall areas and from warm, humid to dry, cold environments (Figure 1). It is possible that the complex nature of the plant's genome, which provides great plasticity to the crop, plays a role in this wide adaptation. Wheat is a C3 plant and therefore thrives in cool conditions.

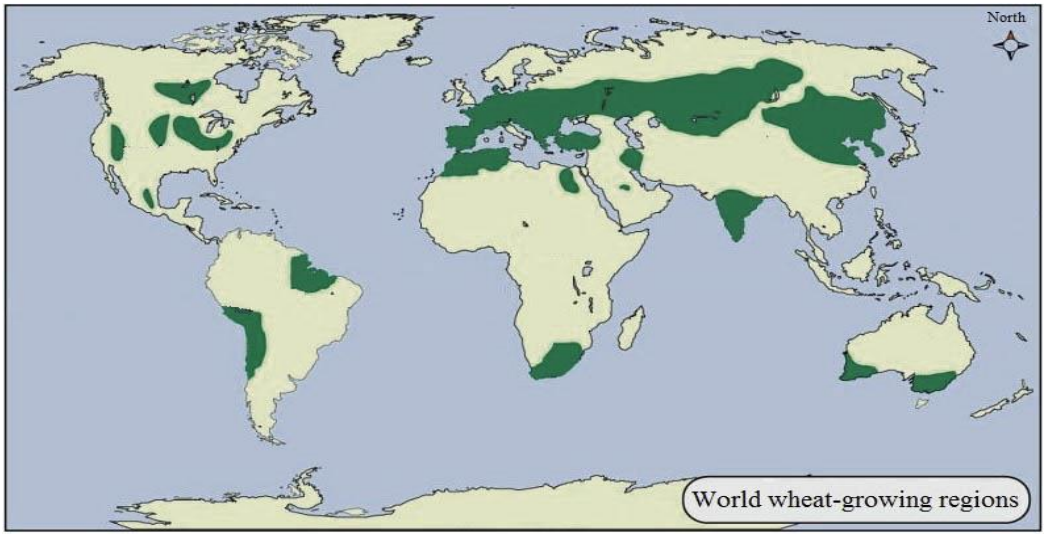

Figure 1. World wheat-growing regions, represented by the dark green colour.

Wheat is a key component of global food security- and provides 20% of the total calories consumed worldwide (SHAHINNIA et al. 2016; NAGY et al., 2018). Global production of this crop over the last decade ranged from 658.64 million tonnes in 2012 to 764.94 million tonnes in 2020 according to the Food and Agriculture Organization of the United Nations (FAO) (FAOSTAT 2020, Figure 2).

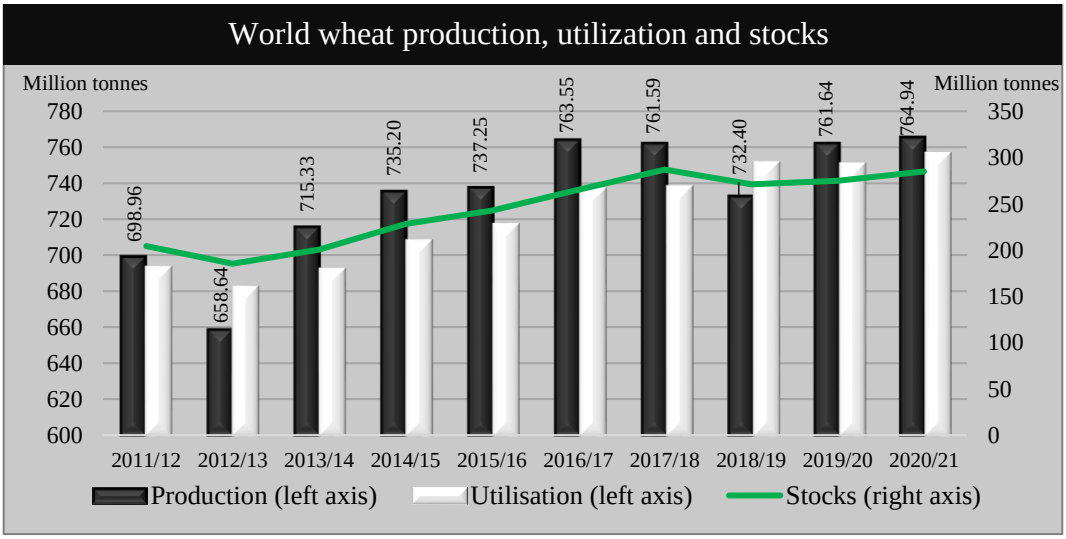

Figure 2. World wheat production, utilization, and stocks over the last decade according to the Food and Agriculture Organization of the United Nations (FAO).

Various wheat experiments have been carried out worldwide to improve its agronomic properties, grain quality, and resistance to different biotic and abiotic stresses.

Due to recent developments in various fields of natural sciences in general and agricultural sciences in particular, researchers are expected to find developed breeding methods that assist to produce new wheat lines or varieties in a short time, with less effort and cost. These new varieties are urgently needed to meet the demands of the growing population and the challenges of climate changes. The conventional cereal breeding method takes between eight and twelve years and highly depends on the environmental conditions. Therefore, breeders do their utmost to find new technologies that make the breeding process more efficient, i.e. molecular marker technology has the opportunity by achieving a wide range of novel goals to improve selection strategies in cereal breeding programmes (WIJERATHNA et al. 2015**)**. *In vitro* doubled haploid method is important in obtaining new wheat lines during a single generation.

Advanced agricultural systems fortified with the use of outstanding varieties, adapted even to stressed environmental conditions such as drought, can be the appropriate long-term way of overcoming the problem of the deterioration of agricultural production due to insufficient water resources. In areas where the prevailing temperatures enable plant growth, the availability of water is one of the most important environmental factors for the productivity of the plants. Plant growth rates are proportional to the amount of water available during the growing season. Due to the importance of water and its vital role in plant metabolism at the cellular and plant level, any decline in water availability has a direct impact on plant growth, and on many biochemical processes, from photosynthesis to photo-assimilated translocation. In dry environments, water molecules are usually strongly adhered to soil particles, and as a result, the amount of absorption water is much lower than the amount of transpiration water, which leads to permanent wilting and can kill plants by dehydration and greatly reduce the water content of plant cells. In order to escape death and ensure life and survival, plants must possess or develop a morphological or physiological mechanism in which they can live under water scarcity conditions and maintain an appropriate growth rate even under harsh environmental conditions.

Thus, the deterioration of wheat yields due to drought problems, lack of water, rainfall stop, or lack of irrigation water or its invalidity due to the high concentration of soluble salts should be overcome by developing drought-tolerant genotypes with good yields.

2. OBJECTIVES

2.1. Characterization of winter wheat genotypes for drought tolerance

- Nine selected genotypes consisting of both drought-tolerant and sensitive wheat varieties and doubled haploid lines – previously tested in various phenotyping trials (NAGY et al. 2017, NAGY 2019) – were studied. Their performance was investigated under well-watered and drought stress treatments with regards to the traits: heading time, plant height, above-ground biomass, main spike length, spikelet number/plant, fertile spikelet number/plant, grain number/plant, grain yield/plant, harvest index, 1000-grain weight, root length, and root dry mass.
- Development of drought-tolerant genotypes that are high yielding to overcome the deterioration of wheat yield due to drought problems caused by a number of factors, such as lack of water, lack of rainfall and irrigation water and the latter exhibiting non-validity due to the high concentration of soluble salts.
- The selected drought-tolerant genotypes will be included into other wheat drought tolerance programmes for investigating their performance as well.

2.2. Generation of winter wheat doubled haploid lines via *in vitro* anther culture

- The main aim of this study was the production of winter wheat (*Triticum aestivum* L.) homogeneous lines via *in vitro* androgenesis for a drought tolerance breeding programme.
- Winter wheat anther culture protocol according to PAUK et al. (2003) with some modifications [cold pre-treatment of donor tillers is in the light (previously in the dark), W14mf is used as induction medium (P-4mf previously used), use of boxes instead of small tubes] was tested on a breeding material comprising 13 different F_4 crossing combinations.
- The effect of the combination (genotype) factor on the androgenetic parameters, such as embryo-like structures, regenerated plantlets, green plantlets, albino plantlets, and transplanted plantlets was identified.
- The doubled haploid lines generated in this project will be assessed in a subsequent programme for drought tolerance and agronomic traits for the release of genotypes and breeding sources.

3. LITERATURE REVIEW

3.1. Origin of wheat species and classification system

Wheat (*Triticum* spp.) belonging to the family Gramineae (Poaceae), genus *Triticum* includes several cultivated species, among which the soft hexaploid wheat (*Triticum aestivum* L.) also called common wheat or bread wheat is the most widely cultivated in the world. About 95% of wheat grown today is hexaploid, and the main use is for making bread and other bakery products (MOHAMADI-JOO et al. 2015). The spelt wheat (*T. spelta*) cultivated in limited quantities is closely related to the common wheat, and sometimes is considered to be one of its subspecies (*T. aestivum* ssp. *spelta*). The hard tetraploid wheat (*T. turgidum* var. *durum*) is the second widely-cultivated today. In addition, the tetraploid wheat emmer (*T. dicoccon*) and the diploid wheat einkorn (*T. monococcum*) are cultivated wheat species (MORRIS and SEARS 1967). It was assumed that the diploid species *T. urartu* (= *T. monococcum*) (2n= 2x= 14, AA) is the donor of the (A) genome set, while the wild tetraploid species (*T. turgidum* var. *dicoccoides*) (2n= 4x= 28, BBAA) is the result of a hybridization between the diploid species *T. urartu* (= *T. monoccum*) and another wild diploid species (*Aegilops speltoides*). The hexaploid cultivated wheat (*T. aestivum*) (2n= 6x= 42, BBAADD) arises from the hybridization between one or more tetraploid species, the wild emmer wheat (*T. turgidum* var. *dicoccoides*) (2n= 4x= 28, BBAA) or durum wheat (*T. turgidum* var. *durum*) (2n= 4x= 28), with wild diploid species (*T. tauschii*) (2n= 2x= 14, DD) (JAUHAR 2007). Figure 3 shows the relations between the cultivated wheat species.

Many classification systems have been used by breeders and farmers for wheat varieties within a species, just to mention the traits growing season, protein content, gluten protein quality and grain colour as a basis.

- Growing season: winter wheat is planted in autumn and is harvested in late spring/early summer, about 80% of cultivated wheat follows this variety, while spring wheat is sown in spring and is harvested in late summer/early autumn (BRIDGWATER and ALDRICH 1966).
- Protein content: the protein content is about 10% for soft wheat with high starch content and about 15% for hard wheat.
- The quality of the wheat protein gluten: this protein can provide wheat special properties for commercial purposes. Strong and elastic gluten, which is suitable for making bread, can trap carbon dioxide during leavening (acidification) in dough bags; the gluten protein in durum wheat is strong but not elastic and is used in pasta, biscuits, cakes, and pastries.
- Grain colour (red, white, or amber): phenolic compounds present in the bran layer change to pigments by browning enzymes. These pigments are responsible for the reddish-brown colour

of wheat, while the content of phenolic compounds and browning enzymes is low in white wheat, which is less astringent in taste than red wheat.

Durum wheat grains contain a carotenoid pigment called lutein, which is oxidized by enzymes in the grains, thus transform into colourless form resulting in the yellowish colour of this wheat and semolina flour.

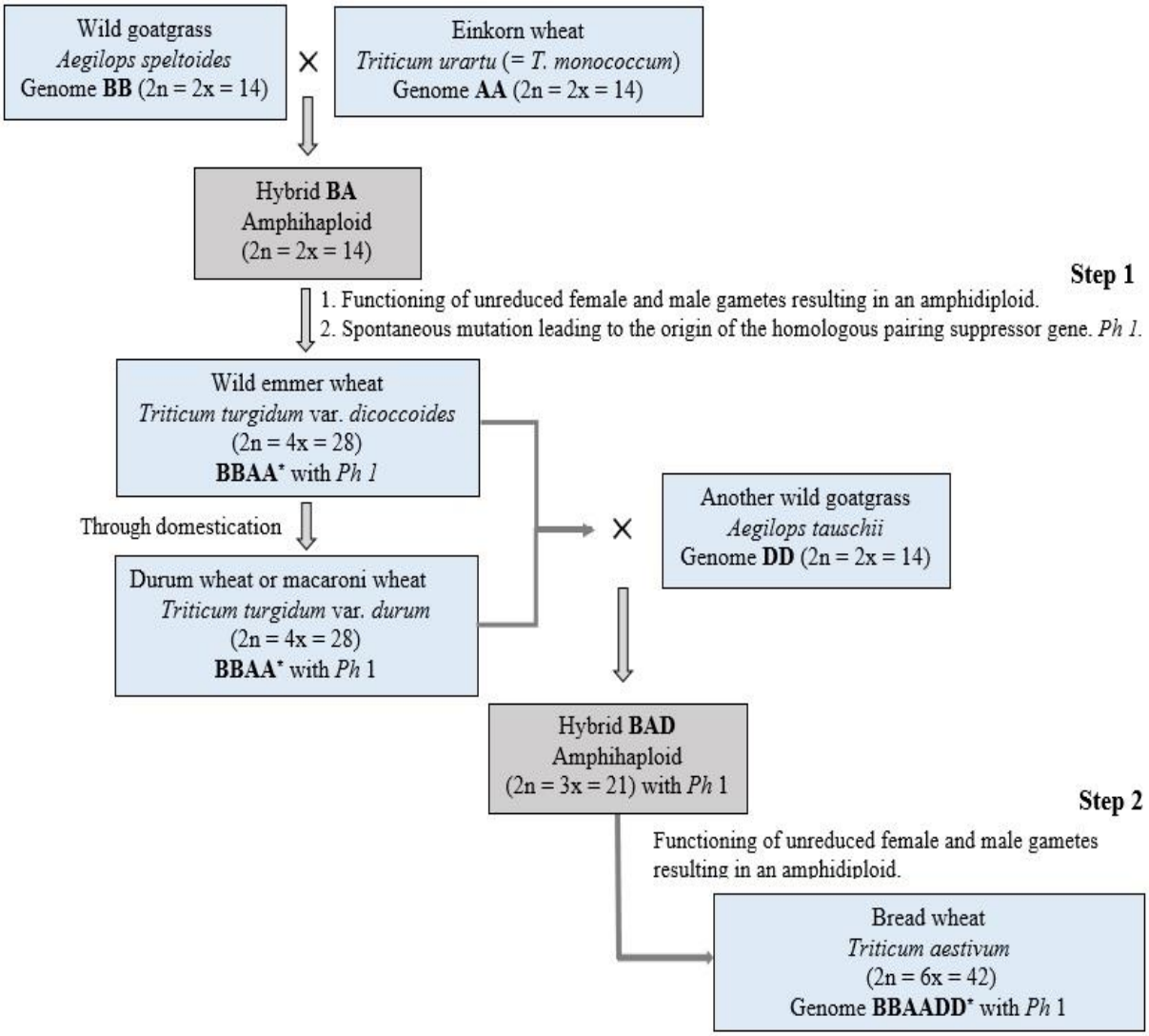

Figure 3. The evolution of bread wheat. (*) The corrected genomic designation for durum wheat is BBAA and that for bread wheat BBAADD, as the cytoplasm donor of the wheat was the B genome (WANG et al. 1997).

3.2. Characterisation of winter wheat genotypes for drought tolerance

The availability of water is one of the most important environmental factors determining the distribution of plant species, in addition to the role of temperature in this aspect. For cereals grown in semi-arid and semi-humid regions, rainfall rates and their distribution play an important role in the productivity. Cereal production declines when the plant is unable to meet all of its water needs during the growth and development stage from germination to maturity. Water stress is caused by an imbalance between the amount of available water and the amount of water required for the plant. Stress effects depend on the plant developmental stages, as some physiological processes in the plant are less sensitive to water deficiency. The success of rain-fed cereal cultivation is mainly determined by the adequacy of rainfall and its distribution during the growing season, besides the water content of the soil.

3.2.1. Drought effects on the morphological traits of plants

The twenty-first century continues to witness realities of climate change, such as elevated temperature, resulting in the occurrence of drought episodes, which are one of the environmental factors that reduce the cereal crop productivity worldwide (TUBEROSA 2012; RAMYA et al. 2016). This further compounds the challenge of global food production where 70% more is needed to feed the rapidly increasing population in spite of the stagnated or declining productivity of crops needed to meet this demand. The declining productivity is in most of the cases due to abiotic stresses (PARIHAR et al. 2015), which cause physiological and biochemical changes during plants' life cycle (KOCHEVA et al. 2013). Negative effects on survival, biomass production and accumulation, and grain yield of most crops thereby occur (GROVER et al. 2001).

Yield decline during drought stress conditions depends on the drought severity, duration of exposure to drought and timing of drought occurrence. Namely, drought effects depend on their occurrence during the phase of plant development (KHAKWANI et al. 2011). Besides, the degree of susceptibility of plants to drought varies both between species and within species depending on the stages of plant development (GROVER et al. 2001) and the interaction between different stress factors (drought, heat, salt, etc.) (BALLA et al. 2012; NAGEL et al. 2015).

Drought factor has many effects on the entire plant level; these include reduced grain germination and seedling formation, poor seedling vigour, reduction of root length, shrinkage of leaves, reduced pollen viability, leaf senescence, incomplete grain filling and reduction of grain yield (PAREEK et al. 1997; SINGLA et al. 1997). High temperatures during the grain-filling period of wheat and barley cause terminal drought that usually occurs in Mediterranean environments (ARAUS et al. 2008). In arid and semi-arid areas, drought is the outstanding obstacle to agricultural productivity leading to accelerated leaf senescence, minimising leaf area,

decreasing photosynthesis and reducing yield (RIVERO et al. 2007). Many studies showed that root growth was less drought-affected than shoot growth, and root sensitivity to drought varies depending on plant species (WESTGATE and BOYER 1985; SHARP et al. 1988).

3.2.2. Plant adaptation and the response to drought

Many factors can affect plant responses to drought stress, such as plant genotype, growth stage, severity and duration of stress, and physiological growth process (CHAVES et al. 2003; NEZHADAHMADI et al. 2013). Adaptation changes and/or deleterious effects are involved as plant reactions to drought; plants can face drought with combinations of various strategies such as avoidance and tolerance (LEVITT 1980; EPSTEIN and BLOOM 2005), which vary with the genotype (CHAVES et al. 2002), for instance, a high growth rate during the wet season with a relatively short lifecycle is one of the important strategies in the arid regions to avoid drought effects. Closing stomata also aids to avoid drought in these regions through reducing water loss due to evaporation, adjusting sink/source allocation by increasing root growth, and reducing canopy through reducing growth and shedding of older leaves, in another way, increasing root/shoot ratio plays an important role in avoiding drought (FISCHER and TURNER 1978). For perennial plants, decreasing the canopy size, which is naturally caused by accelerated leaf senescence and leaf abscission during drought stress, maintains the survival of the plant and completion of the plant lifecycle under drought, but this strategy contributes to reducing the yield of the annual crops and causes economic loss to farmers (RIVERO et al. 2007).

LEVITT (1972) formulated an important and rational classification of plant resistance to drought; it was the best among all definitions provided by others in recent years. This classification was based on mechanisms or strategies that allow the plant to mitigate the harmful effects of water deficiency in the soil. Accordingly, strategies were grouped into two broad categories: dehydration avoidance and dehydration tolerance. In this aspect, the combination of morphological-physiological features that allow plants or parts of them to maintain hydration is defined as dehydration avoidance, e.g., deep roots, early flowering, deposition of waxes, osmotic adjustment, etc.

On the other hand, mechanisms or features that enable plants to maintain their proper function at least partially under highly dehydrated conditions are classified under dehydration (desiccation) tolerance, e.g., remobilisation of stem water-soluble carbohydrates, accumulation of molecular protectants, etc. (TUBEROSA 2012).

Stress perception, signal transduction, transcriptional activation of stress genes, synthesis and accumulation of stress proteins are some of the ways in which plants respond to drought stress, thereby bringing about biochemical, cellular and physiological manifestations (GROVER et al.

2001). Changes in the phenotype and partitioning (redistribution) of dry matter can occur in plants that respond to such stress (PASSIOURA 2012), e.g., small leaf area, small plants, reduced leaf area or increased root biomass (increased root density and length), thereby these manifestations contribute in mitigating of damages resulting from drought (RICHARDS et al. 2010; WANG et al. 2017).

3.2.3. Breeding and phenotyping for drought resistance

3.2.3.1. Important phenotyping traits for wheat drought tolerance

Drought tolerance, if taken as a concept, generally refers to the plant's ability to maintain yield under water-limited conditions (HOFFMANN and BURUCS 2005), whereas from an agronomic point of view, it can be interpreted as a plant's ability to reduce yield loss due to scarcely available water (CLARKE and MCCAIG 1982). The characterization is still the main criterion for the study and selection of drought-tolerant breeding materials based on drought-adaptive and constitutive morpho-physiological traits with grain yield and its components among these traits. Therefore, phenotyping leads to an understanding of the drought adaptation responses of the plant species (PASSIOURA 2012; DEL POZO et al. 2016; NAGY et al. 2018). Knowledge of the phenotype response of plants is urgently needed in breeding programmes to release high and stable yields and thus be better prepared, considering climate change's threat to food security (BROWN et al. 2014). Plant researchers have endeavoured to provide appropriate strategies of plants that will be able to withstand the environmental stress, insects, and diseases and maintain a high yield under stress conditions (AHMED et al. 2013). Researchers have developed reliable, automatic, and high-throughput phenotyping programmes to meet the needs of current research (HARTMANN et al. 2011).

For a successful breeding programme, methods for phenotypic characterization of drought-tolerant genotypes within a large number of wheat genotypes should be easy, rapid and somewhat cheap (GRZESIAK et al. 2019). Morphological and physiological traits enabling the wheat to grow under water deficiency and to provide grain yields were specified (HOFFMANN and BURUCS 2005). Thus, the characterization of the basic breeding materials in addition to performance under optimal and drought conditions is an essential process in breeding for drought tolerance.

The shoot dry weight and yield parameters measured after harvest are relevant traits in the characterization of wheat genotypes for drought tolerance (MAJER et al. 2008). Furthermore, the importance of root traits for drought tolerance has been well confirmed (WASAYA et al. 2018), where the effects of water shortage on plants will eventually lead to an increase in root growth

(KEIM and KRONSTAD 1981). Many studies have revealed the role of the deep and strong root systems for higher yields in wheat (MANSCHADI et al. 2010; WASSON et al. 2012), barley (FORSTER et al. 2005) and other cereal crops, while some rice-conducted experiments showed a notable lack of correlation between root features and drought tolerance (PANTUWAN et al. 2002; SUBASHRI et al. 2009).

The roots are characterised by a spectacular level of morphological plasticity in response to the physical soil conditions (FORDE 2009; NAGY et al. 2018). This peculiarity enables plants to better adapt to the chemical and physical properties of the soil, especially under water-limited conditions (YU et al. 2007). The root system architecture is influenced by many factors such as temperature, moisture, nutrients and soil pH (ROBBINS and DINNENY 2015). Root size and architecture have an important effect on the final yield that will rely on the distribution of soil moisture and the level of competition for water resources within the plant community (KING et al. 2009; WASAYA et al. 2018). Thus, selection for faster-growing and deeper roots is an effective choice for breeders to enhance water harvest and improve yield stability under water deficit conditions in case of additional stored moisture is present in deeper soil layers.

Flowering time is another critical factor for an ideal adaptation that affects the yield in environments with limited water availability and distribution during the growing season (TUBEROSA 2012). Crop ability to decrease the days to heading and the days to maturity may ensure a drought escape. A number of experiments that applied different water availability levels on various crops confirmed the relationship between the plasticity of yield and flowering time (SADRAS et al. 2009).

Evaluation of the yield performance of genotypes in diverse environments with varying water availability – well-watered, moderate water lack and severe drought – allows effective prediction of the drought resistance of genotypes (MOHAMMADI 2016). Therefore, phenotyping using controlled water regimes provides yield-based screening, enabling the selection of genotypes with high yields under both well-watered and drought stress conditions (MWADZINGENI et al. 2016a). The relative yield performance of genotypes under drought stress and well-watered conditions is considered as an essential onset point to identify the traits associated with drought resistance and the selection of genotypes that tolerate drought stress (SIO-SE MARDEH et al. 2006). FERNANDEZ (1992) divided the genotypes into four groups according to their yield response to stress conditions (group A): genotypes having high yield under well-watered and stress conditions, (group B): genotypes with high yield under well-watered conditions, (group C): genotypes with high yield under stress conditions and (group D): genotypes producing low yield under both well-watered and stress conditions.

A group of target traits associated with yield under stress conditions have been pinpointed for drought tolerance (MWADZINGENI et al. 2016b), i.e.: reduced plant height related to the high harvest index (SLAFER et al. 2005), reduced number of days to anthesis and maturity, which enables plants to avoid terminal drought stress (BLUM 2010), and root architectural traits, i.e., longer, dense, and distributed roots, which effectively aid plants to uptake water from deeper soil layers (EHDAIE et al. 2012).

3.2.3.2. Irrigation systems in the phenotyping of wheat drought tolerance

Researchers have developed various methods for phenotyping drought tolerance in wheat, some of them have chosen the field under rain-fed conditions (MOHAMMADI-JOO et al. 2015; AL-SALIMIYIA et al. 2018), or pots inside a rainout shelter in the field (WANG et al.2017), others preferred greenhouse conditions (GÁSPÁR et al. 2005; MAJER et al. 2008; NAGY et al. 2018), while many researchers have conducted their characterization under *in vitro* conditions (RAZMJOO et al. 2015). Physiologists and breeders have applied various irrigation regimes during the wheat lifecycle, in which some wheat plants have been irrigated to 65–70% field water capacity under well-watered conditions and 30–35% field water capacity under drought stress conditions (GRZESIAK et al. 2019). MWADZINGENI et al. (2016a) ceased watering at 35% field water capacity to cause stress conditions before wheat re-irrigation, while others, in their experiments, applied 20% soil moisture capacity to create drought stress conditions and 60% soil moisture capacity under controlled conditions (NAGY et al. 2017, 2018). In the experiment carried out by ABID et al. (2016) to study the effects of moderate drought stress on wheat, the irrigation was applied to 55-60% of field capacity as water stress treatment, and 80% field capacity as well-watered treatment, while WANG et al. (2017) exposed wheat to three irrigation regimes: well-watered conditions (80% field water capacity), moderate drought stress (50% field water capacity), and severe drought stress (25% field water capacity) from 30 days after sowing to maturity.

3.2.3.3. Selection methods of wheat drought tolerance

Researchers and breeders differed in the pattern of selection under the different environments. Some researchers preferred selection under non-stress conditions (BETRAN et al. 2003), others opted for selection under stress conditions (MOHAMMADI et al. 2011), while several others decided to choose mid-way and believed in selection under both non-stress and stress conditions (SIO-SE MARDEH et al. 2006; NAGY et al. 2018). Moreover, different breeding programmes on wheat aimed to apply selection to improve the quantity, quality, and stability of yield under drought stress for the development of new drought-adapted genotypes (GRZESIAK et al. 2019). Genotypes that achieve relatively high yields under both stress and non-stress conditions

should be targeted during selection in order to ensure adaptation to drought conditions (MWADZINGENI et al. 2016b). The desired traits for improving yield in water-limited environments must be genetically correlated with yield and have a higher heritability than the yield itself (BLUM 2018). In water-limited environments, the pattern of biomass allocation is an important adaptive strategy in wheat. The accumulation and allocation of biomass are closely linked to the size of the crop organs and the plant architecture (WANG et al. 2017). TAHMASEBI et al. (2013) reported that the selection of better genotypes with desirable yield, in addition to the use of yield-associated traits in the breeding programme and the identification of ideal selection criteria, are convenient ways for a successful genotyping programme.

3.3. Generation of winter wheat doubled haploid lines via *in vitro* anther culture

3.3.1. Concept and importance of anther culture method in breeding

Currently, most wheat breeding programmes aim to obtain new varieties characterised with high-yielding, excellent grain quality, good nutrient responses, and resistance to biotic and abiotic stress factors. Plant breeders endeavour to achieve this goal quickly by integrating biotechnology methods with traditional breeding techniques, thus saving cost and efforts as well.

In vitro anther culture is one of the efficient biotechnology methods in plant breeding of wheat to produce doubled haploid lines from immature pollen grains (microspores) in anthers. However, it can be adopted by breeders only if it ensures obtaining a sufficient rate of the double haploid plants from a wide range of wheat genotypes (BARNABÁS et al. 2001; TRIGIANO and GRAY 2016).

The success of anther culture method is associated with producing a high number of embryo-like structures, green plantlets and doubled haploid lines. Low rate of embryo-like structure formation, green plantlet regeneration, and doubled haploid line production in several wheat genotypes limits the use of anther culture in wheat breeding programmes.

In nature, the original pathway of microspore development (gametophytic pathway) in anthers leads to the formation of male gametes required for double fertilization. In *in vitro* anther culture method, some of the microspores present in anthers can reprogramme their original developmental pathway under specific stress conditions following a new sporophytic pathway of development involving continuous divisions. As a result of these divisions, haploid embryo-like structures or calli are induced. This process is known as the androgenesis, which can be formed in various higher plants, including cereals (HEBERLE-BORS 1985).

During sporophytic development, embryo-like structures are formed after symmetrical divisions of microspores, while the formation of calli occurs after the further division of the

vegetative-typed cells resulting from the asymmetrical division of microspores. This was proved by the results of the analysis using a transmission electron microscope (BARNABÁS et al. 1988).

Haploid plants that contain a gametic chromosome number (n) can arise from microspores in anthers in the process of androgenesis or an egg cell by gynogenesis, but they can arise from a gametophytic cell other than the egg cell, too, in this case, it is called apogamy. Besides, they can be obtained from a spontaneous development or the hybridization process.

BLAKESLEE et al. (1922) wrote the first report on the spontaneous development of the haploid *Datura stramonium*. The first discovery of haploid breeding occurred in 1964 when GUHA and MAHESHWARI performed a haploid embryo formation from an *in vitro* culture of *Datura* anthers. This was shortly followed by a successful *in vitro* haploid production of tobacco (NITSCH and NITSCH 1969). Since then, many successful efforts have been made to obtain haploids from different species, and by 2003 more than 250 protocols covering almost all families in the plant kingdom have been published (reviewed by MALUSZYNSKI et al. 2003).

In cereal crops, the application of the doubled haploid technology enables genetically the realisation of homozygous pure lines from heterozygous breeding material in one generation (YAN et al. 2017). Improvements and the adoption of the technology have rendered it a fast alternative to the conventional breeding methods and it has become an indispensable method in the attainment of homogeneity in different researches and programmes (WĘDZONY et al. 2009; LANTOS and PAUK 2016; MAHATO and CHAUDHARY 2019). The technology also assists in more accurate assessment of QTL × environment interactions (YAN et al. 2017) and was used in genetic studies for marker-trait association researches (SORRELLS et al. 2011), genomics and as a target for transformation (MUROVEC and BOHANEC 2012), genetic engineering (RAVI and CHAN 2010), mapping of genes (HAO et al. 2013), and mapping of quantitative trait loci (QTLs) (SHI et al. 2019).

The main methods applied in breeding to produce doubled haploid lines involve wide hybridization, gynogenesis, and androgenesis (DUNWELL 2010). Intergeneric hybridization, i.e., crossing with maize (*Zea mays* L.) or *Hordeum bulbosum* (SUENAGA et al. 1997), anther culture (CASTILLO et al. 2015), and isolated microspore culture (LIU et al. 2002) are the most-known and used methods for the doubled haploid production in winter wheat (*Triticum aestivum* L.) and different cereals (LANTOS and PAUK 2016). Anther culture is effective and appropriate, enabling the production of several haploid plants from an individual anther. Other cereal crops for which protocols for doubled haploid have been used include barley, triticale, rice, maize and rye (FLEHINGHAUS et al. 1991; IMMONEN and TENHOLA-ROININEN 2003; DUNWELL 2010; NIU et al. 2014). Using this approach to plant improvement, researchers have produced registered cultivars (KUSH and VIRMANI 1996) and commercial varieties (THOMAS et al. 2003).

In winter wheat, *in vitro* anther culture has been successfully applied in various research programmes to release new varieties, i.e., ‘Jinghua No-1’ (HU et al. 1986), ‘Florin’ (DE BUYSER et al. 1987), ‘GK Délibáb’ (PAUK et al. 1995), ‘McKenzi’ (GRAF et al. 2003) or ‘AC Andrew’ (SADASIVAIAH et al. 2004).

Various factors affecting the androgenetic production efficiency by anther culture include genetic background of donor plants (KONDIC-SPIKA et al. 2011), the collection timing of tillers, which mirrors the microspore developmental stage (HE and OUYANG 1984), the physiological growth circumstances of plants (EL-HENNAWY et al. 2011), different abiotic pre-treatments (ISLAM and TUTEJA 2012), physical factors in tissue culture such as, light and temperature; and composition of anther culture medium (BROUGHTON 2008; ŻUR et al. 2015).

3.3.2. Growing conditions and collection time of donor plants

Donor plants affect the efficiency of *in vitro* androgenesis in anther culture, thus also the final doubled haploid production. Donor plants could be grown under two conditions: controlled (greenhouse, phytotron chamber) and non-controlled (field, nursery).

Controlled light and temperature conditions provide the possibility for growing donor plants throughout the year (PAUK et al. 2003; SORIANO et al. 2007, 2008; BROUGHTON 2008, 2011; CASTILLO et al. 2015; COELHO et al. 2018; ORLOWSKA et al. 2020; BROUGHTON et al. 2020). Therefore, the plant materials for anther culture improvements and applied research are not restricted to certain months.

Donor plants growing under optimal growing conditions (temperature, light, and humidity) provide healthy tillers and spikes that are the onset for doubled haploid production.

The winter wheat genotypes require a vernalisation period of 6–8 weeks at 3–4°C after germination. The common conditions for healthy plants are controlled at approximately 18–21°C/day and 12–15°C/night with 12–18 h photoperiod and 70–80% humidity (SORIANO et al. 2007, 2008; SANCHEZ-DIAZ et al. 2013; CASTILLO et al. 2015; COELHO et al. 2018; BROUGHTON et al. 2020). In addition, the donor plants are regularly nourished with a fertilizer solution.

Many researchers, in their experiments, e.g. PAUK et al. (2003); LANTOS et al. (2013); WEIGT et al. (2016, 2019, 2020); LAZARIDOU et al. (2016), have utilized field-grown donor plants that produce more tillers with bigger spikes, more anthers and microspores within anthers. This positively affects the number of androgenetic embryo-like structures and green plantlets, and thus generates a relatively high rate of doubled haploid plants for practical breeding programmes and applied research.

In order to ensure an efficient anther culture technique and induce the androgenesis of *in vitro* wheat anther culture, donor tillers should be harvested when the developmental stages of the microspores in anthers (uninucleate vacuolated microspores) are at a narrow range, namely, at early-, mid-, or late-uninucleate stages. In anther- and isolated microspore culture of wheat, the microspore embryogenic process was induced and tracked to investigate the development, that is, the initial cell division and embryo formation of microspores (INDRIANTO et al. 2001; DATTA 2005; DWIVEDI et al. 2015; SELDIMIROVA et al. 2017; NIAZIAN and SHARIATPANAHI 2020). According to previous publications, most researchers isolated anthers containing microspores at mid- to late-uninucleate stages (SORIANO et al. 2007, 2008; BROUGHTON 2008, 2011; REDHA and SULEMAN 2011; RUBTSOVA et al. 2013; CASTILLO et al. 2015; WEIGT et al. 2016, 2019, 2020; LAZARIDOU et al. 2016; BROUGHTON et al. 2020; ORLOWSKA et al. 2020). While other researchers isolated anthers with microspores at early- and mid-uninucleate stages to induce androgenesis in wheat anther culture, e.g. PAUK et al. (1995); TUVESSON et al. (2000, 2003); DATTA (2005); LANTOS et al. (2013); LANTOS and PAUK (2016); KANBAR et al. (2020). Results of the androgenetic production of isolated anthers with microspores at the early- and mid-uninucleate stages were more efficient (PAUK et al. 1995; LANTOS et al. 2013; LANTOS and PAUK 2016; KANBAR et al. 2020).

3.3.3. Albinism incidence

Several studies have found that doubled haploid production in wheat is limited by albinism incidence (ISLAM 2010; BROUGHTON 2011; LANTOS et al. 2013).

Albinism induced by androgenesis in anther cultures is genetically conditioned (ZAMANI et al. 2000; MAKOWSKA et al. 2015), and appears in the plantlets when the proplastids become unable to transform into chloroplasts (MAKOWSKA and OLESZCZUK 2014).

Many complex factors can contribute to this incidence, such as altered transcript patterns and translation levels (ANKELE et al. 2005), deletions and reorganization of plastid genomes (DAY and ELLIS 1985) and the maternal inheritance of plastids (VAUGHN et al. 1980).

Albinism occurs in androgenesis-derived plants in the majority of cereals (wheat, barley, rye, triticale, rice and oat). The rate of albino plantlets in cereals may range from 5–100% of regenerated plantlets. Within the same species, there was a variation among genotypes in respect of albinism (MAKOWSKA and OLESZCZUK 2014; KRZEWSKA et al. 2015). That was proved in the experiment conducted by WEIGT et al. (2016) when they compared the androgenetic capability of solid, medium and hollow-stemmed wheat genotypes by *in vitro* anther culture method. They concluded that the solid-stemmed genotypes generated higher frequency of albino

plantlets on the medium with 2,4-D (2,4-dichlorophenoxyacetic acid) and kinetin, while hollow-stemmed genotypes yielded more albino plantlets on the medium containing 2,4-D and dicamba.

Various trials were carried out to overcome the albinism incidence in anther culture during induction of doubled haploid plants by anther culture. The use of copper sulphate (JACQUARD et al., 2009), *n*-butanol treatment (SORIANO et al. 2008; BROUGHTON 2011), co-culture of ovaries (BROUGHTON 2008), and polyamine treatments (REDHA and SULEMAN 2011) had positive effects on the number of green plantlets and negative effects on the number of albino plantlets.

Although many researchers have reported that albinism in cereal crops is a heritable trait and nuclear genomes control over this incidence (LANTOS et al. 2013; HASAN et al. 2014; KRZEWSKA et al. 2015), the interaction between genetic factors and other affecting factors such as pre-treatment of anthers, collecting time of donor plants and physical factors may increase this incidence as well.

3.3.4. Genotype dependency

Genotype dependency is the main obstacle to doubled haploid wheat production via *in vitro* anther culture (ISLAM 2010; BROUGHTON 2011; LANTOS et al. 2013; KANBAR et al. 2020); the genotypic impact on the response to anther culture limits the effectiveness of the anther culture method for breeding purposes (TUVESSON et al. 2000; CHEN et al. 2011; KONDIC-SPIKA et al. 2011; DWIVEDI et al. 2015).

The response of wheat to androgenetic induction by anther culture differs depending on the genotype, among species, and even within species. For example, for hexaploid wheat, it has been reported that winter genotypes are more responsive than spring ones (SHARMA et al. 2005). There were different results in the experiment conducted by ZAMANI et al. (2000). They showed that embryo-like structures induced by anther culture were more efficient in winter wheat genotypes compared with spring ones. However, green plantlet regeneration from the spring genotypes was much higher than from the winter ones. CHAUDHARY et al. (2003) investigated the androgenetic production of nine elite winter wheat genotypes and two spring wheat genotypes by anther culture method. Their results revealed that the spring genotypes produced a higher number of embryo-like structures and green plantlets. GRAUDA et al. (2014) studied the androgenetic induction of sixteen winter wheat hybrids and five spring ones. They proved that the spring wheat hybrids yielded higher embryogenesis than the winter ones, but the winter wheat hybrids had higher green plantlet regeneration rates compared with the spring ones. HOLME et al. (1999) discovered in their studies, that the wheat genotypes of North-western European origin are less responsive than their Eastern European counterparts. LAZARIDOU et al. (2016) compared

the frequencies of embryo-like structures and green plantlet regeneration of bread wheat with their extracted tetraploid (BBAA) when they applied three different pre-treatments: cold pre-treatment for 7 and 18 days at 4°C, and 0.3 M mannitol for 7 days at 4°C. W14 and 190-2 were used as the induction and regeneration media, respectively. Their results showed that the androgenetic response per three treatments in winter wheat genotypes was better compared with the extracted tetraploid wheat. Furthermore, no green plantlets per all pre-treatments were obtained from tetraploid wheat, and the results proved the role of D genome in anther culture androgenetic response in wheat. Thus, hexaploid wheat (*T. aestivum* L.) is characterised as well-responding in anther culture and has been used widely and successfully (KASHA and MALUSZYNSKI 2003), whereas the efficiency of anther culture in durum wheat (*T. turgidum* L.) was slight and almost no green plantlets were produced due to lack of D genome (CISTUÉ et al. 2009; LAZARIDOU et al. 2016). However, possible interactions between the three genomes of hexaploid wheat (*T. aestivum* L.) may stimulate the androgenetic response in anther culture.

3.3.5. Increase of wheat anther culture efficiency

In vitro anther culture system has been efficient only for a restricted range of responsive genotypes, and other genotypes are still non-responsive. Hence, more effective methods are demanded to stimulate androgenesis in a wide range of wheat genotypes.

Genotype dependency and albinism are the most limiting factors for doubled haploid production via anther culture (ISLAM 2010; BROUGHTON 2011; LANTOS et al. 2013). For that reason, factors mitigating both genotypic dependency and albinism incidence should be identified to improve wheat anther culture efficiency.

3.3.5.1. Genetic improvements

Several kinds of research have been conducted in recent decades to improve the efficiency of wheat anther culture by genetic improvements. For example, TUVESSON et al. (2000) presented a strategy to achieve this purpose through using responsive breeding materials in crossing. The success of this strategy depends on the precondition, that one parental plant material in each cross should be previously tested in anther culture and should produce at least one green plantlet/spike (TUVESSON et al. 2003). Use of responsive plant material was also suggested in other breeding programmes depending on anther culture (GONZÁLEZ et al. 2006; KONDIC-SPIKA et al. 2011).

MARCINIAK et al. (2003), DAGÜSTÜ (2008), YILDIRIM et al. (2008), and AL-ASHKAR (2014) have reported that the embryo-like structure formation and green plantlet regeneration in anther culture are inherited traits. CHAUDHARY et al. (2003), DAGÜSTÜ (2008)

and GRAUDA et al. (2016) found that additive, dominant, and epistatic gene influences control the inheritance pattern of the androgenetic traits in anther culture, while some studies have indicated that the androgenetic response follows a simple inheritance pattern and is controlled by dominant genes (EL-HENNAWY et al. 2011) that can be easily transferred y from high responsive genotypes to low responsive ones by crossing process with expected rapid genetic gain.

3.3.5.2. Application of stress pre-treatments in anther culture

In *in vitro* anther culture, most studies aimed at improving of this method concentrate on the application of convenient stress pre-treatments (cold pre-treatments, colchicine, hormones, and other chemical agents) to induce the androgenesis in cereals (LABBANI et al. 2007), where this stress leads to repeated equal divisions of the microspore nucleus, thus reprogramming the microspore developmental pathway from the gametophytic to sporophytic (ZHOU and KONZAK 1997; ZHENG and KONZAK 1999; BARNABÁS et al. 1991; LAZARIDOU et al. 2016; BROUGHTON et al. 2020; WEIGT et al. 2020). The use of pre-treatment should be convenient not to result in high mortality rates of cells or to cripple the cellular function (MAKOWSKA and OLESZCZUK 2014).

Cold pre-treatment of donor tillers is the simple way to re-programme the microspores. *In vitro* androgenesis of microspores can be induced via long cold pre-treatment (2–5°C, 10 days – 4 weeks) of donor tillers (PAUK et al. 2003; LANTOS et al. 2013; LANTOS and PAUK 2016; COELHO et al. 2018; KANBAR et al. 2020). Short cold pre-treatment (3–8 days, 4–6°C) can also be used for induction of androgenesis (BROUGHTON 2008, 2011; RUBTSOVA et al. 2013; WEIGT et al. 2016, 2019; LAZARIDOU et al. 2016).

LAZARIDOU et al. (2016) carried out a research to investigate the role of D genome in the androgenetic response and to study the interaction between genotype and the applied pre-treatments, which were 7-day pre-treatment at 4^{o}C, 18-day pre-treatment at 4^{o}C, and 0.3 M mannitol for 7 days at 4^{o}C. They concluded that tetraploid wheat (*Triticum turgidum* L.) achieved lower induction of embryo-like structures and no green plantlet regenerations per three pre-treatments, as compared with hexaploid wheat (*Triticum aestivum* L.), and the genotypes responded better after 7-day cold-pre-treatment of spikes. However, within hexaploid wheat, the genotypes varied in their androgenetic responses per three treatments, where the Canadian genotypes responded better after 18-day cold-pre-treatment at 4^{o}C compared with the controls, which performed better after 7-day cold-pre-treatment. Besides, the mannitol resulted in a negative influence on both the embryo-like structures in some hexaploid genotypes and green plantlet production in all hexaploid ones. In the investigation of LAZARIDOU et al. (2016), the results revealed that there was a high interaction between the genotype and the cold-pre-treatments of

winter wheat spikes and this contradicted to RIZKALLA et al. (2012) who asserted that winter wheat genotypes had almost the same embryo-like structure induction following cold-pre-treatments of spikes for 7 or 14 days. In most experiments, cold-pre-treatment was applied in several cereal crops for this purpose; TREJO-TAPIA et al. (2002) revealed that the cold-pre-treatment had a vital role in rice for embryo-like structure induction from anthers of the parental lines and the F_1 hybrids.

Various protocols have been proposed concerning the effect of the presence of mannitol during the cold-pre-treatment stage in the androgenetic induction of wheat. In an endeavour by CISTUÉ et al. (2006) to improve durum wheat (*Triticum turgidum* L.) androgenetic response, the results showed that 5-day pre-treatment of the anthers with 0.7 M mannitol had a positive effect on the formation of green plantlets. SORIANO et al. (2007) and CASTILLO et al. (2015) confirmed that the 5 -day pre-treatment of winter wheat (*Triticum aestivum* L.) anthers with 127.5 g/L mannitol resulted in satisfying results of the androgenetic induction. Moreover, LABBANI et al. (2007) confirmed that the interaction between the combination of cold and 0.3 M mannitol pre-treatments of anthers for seven days had a high influence on the embryo-like structure formation and the green plantlet regeneration of the tetraploid wheat (*Triticum turgidum* L.). None of the previous suggestions, however, has been confirmed in the study of LAZARIDOU et al. (2016) due to the negative effects of mannitol on androgenetic induction of durum and winter wheat.

Heat shock treatment of isolated anthers at 32°C for 36 h in the dark was frequently applied in anther culture method as a stress factor to improve androgenesis in winter wheat (OUYANG et al., 1983; PAUK et al., 2003; LANTOS et al., 2013; LANTOS and PAUK, 2016; KANBAR et al. 2020). According to a report by OUYANG et al. (1983), the optimal incubation temperature of isolated anthers after heat treatment was between 28–30°C for cereal crops. Higher incubation temperatures can result in an increased frequency of albino plantlets.

In microspore embryogenesis, SELDMIROVA et al. (2016) and BIESAGA-KOŚCIELNIAK (2001) verified that auxin gradients play an essential role in setting up embryo symmetry and that the accurate ratio of endo- and exogenous auxins in the microspores determines the microspore developmental pathway toward embryo-like structure formation. The type and length of exposure to the stress factor influence the accumulation of endogenic auxins (mainly IAA). Based on this report, it is crucial to select the appropriate primary treatment and the convenient concentration of hormones applied to the induction medium, in particular, which can optimize the total concentration of all auxins inside a cell. The type and concentration of auxins, as well as the type of carbon source, influenced the induction of embryo-like structures (TREJO-TAPIA et al. 2002).

Auxinic herbicide 2,4-D is widely-utilized as a growth hormone for inducing embryo-like structures (PRZETAKIEWICZ et al. 2003; SELDIMIROVA et al. 2016). The previous studies showed that this synthetic hormone added into the induction medium behaves as a stress factor and has auxin-like effects (FEHÉR 2005). Its convenient concentrations in the induction medium should range between 0.5 and 2.0 mg/L for this purpose (WEIGT et al. 2019). Too high concentrations, over 2.0 mg/L, may cause loss of the embryo-like structures' ability to regenerate into plantlets because the stress hormones accumulate and hinder further development of embryos (ZHENG and KONZAK 1999). Too low concentrations of auxin, below 0.5 mg/L, do not stimulate embryo-like structure induction at all (GORBUNOVA et al. 2001).

In studies concerning wheat microspore embryogenesis, different growth hormones were added into induction media as stress factors, such as dicamba, kinetin, picloram (CHAUDHARY et al. 2003; CISTUÉ et al. 2006), PAA (ZIAUDDIN et al. 1992; KIM and BAENZIGER 2005), and BAP (CISTUÉ et al. 2006; PONITKA and ŚLUSARKIEWICZ-JARZINA 2009).

Zearalenone (ZEN) was also applied in the induction medium as a stress factor for embryo-like structure induction; it has auxin-like effects (SZECHYŃSKA-HEBDA et al. 2007; WEIGT et al. 2019). WEIGT et al. (2019) used anther culture method to assess the impact of zearalenone and hormone regulators on microspore embryogenesis concerning 13 F_1 hybrids of winter wheat and six F_1 hybrids of spring genotypes. They applied two combinations of growth hormones: the auxins (2,4-D + dicamba), and auxin and cytokinin (2,4-D + kinetin), each with three ZEN concentrations (0 mL/L, 0.1 mL/L, 0.2 mL/L), thus six combinations of media were formed. The results showed that the media with ZEN caused an efficient increase in embryo-like structures and green plantlet number in some hybrids. In addition, the increased concentration of ZEN improved effectively the microspore embryo-like structure induction. The induction medium (2,4-D + dicamba) supplemented with 0.2 mL/L ZEN was the most effective one. As a result of the use of ZEN together with growth hormones, all hybrids produced embryo-like structures, thus the non-responsive wheat hybrids were stimulated, besides green plantlet regeneration was obtained from 18 out of 19 investigated hybrids. Adding ZEN to the medium did not influence either the number of albino plantlets or the percentage of spontaneous doubled haploid plants.

Colchicine can also increase *in vitro* androgenetic response in wheat, and many researchers have reported that colchicine has a positive effect on embryo-like structure induction or/and green plantlet regeneration, and this effect differed according to the genotype (BARNABÁS et al. 1991; HANSEN and ANDERSEN 1998a; SORIANO et al. 2007). The presence of colchicine in the induction medium caused a significant increase in the frequency of embryo-like structures in wheat (BARNABÁS et al. 1991; BARNABÁS and KOVÁCS 1992). In order to increase the haploid

frequency in anther culture, it could be more efficient to apply low concentrations (0.01, 0.02, 0.04%) of colchicine added into the induction medium (BARNABÁS et al. 1991).

There is a confirmation that the herbicides trifluralin, oryzalin and APM (amiprophosmethyl) had the same effects as colchicine in stimulating androgenesis. The study conducted by HANSEN and ANDERSEN (1998b) showed that trifluralin and APM prompted the embryo-like structure induction in wheat microspores. HANSEN and ANDERSEN (1996) reported that oryzalin, trifluralin and APM prompted the embryo-like structure induction in *Brassica napus* as well. In the mentioned studies, the concentrations of herbicides were applied as 0.1–10 μM with wheat and 0.3–30 μM with *Brassica napus* for 24 and 48 h exposure times. The convenient herbicide concentrations for embryo-like structure induction and green plantlet regeneration ranged between 0.3–1.0 μM, but the higher herbicide concentrations hindered the embryo-like structure formation and green plantlet regeneration in both species. In contrast, the increased concentrations of the used herbicides improved steadily the rate of plant fertility, and thus the doubled haploid plant production. BROUGHTON et al. (2020) studied the effects of trifluralin on the androgenetic induction of wheat and selected 1 and 3 μM concentration, and exposure times of trifluralin were based on the HANSEN and ANDERSEN (1998b) study (24, 48 h). The results showed that no positive effects on the number of embryo-like structures and green plantlets occurred in the application of trifluralin even when a low concentration of 1 μM trifluralin was applied. These observations prove that trifluralin can only be applied with wheat microspore method, not with the anther culture one.

Due to the presence of the strong genotype dependency between spring and winter wheat, it is essential to select the appropriate pre-treatment factors, such as cold-pre-treatment, concentration, and type of hormones in the induction medium adjusted either to the spring or the winter wheat. This was one of the essential solutions to increase the effectiveness of androgenesis (WEIGT et al. 2020). The winter wheat is more tolerant of low temperatures. This fact causes differences in the level of endogenous hormones induced in cells of spring and winter wheat during cold stress, and thus affects the efficiency of androgenesis. WEIGT et al. (2020) studied fifteen winter and fifteen spring wheat genotypes separately by using the microspore androgenesis method and analysed the differences between them in reaction to the hormone content. They applied C17 induction medium supplemented with two combinations of growth hormones; I: the auxins only [1 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D) + 1 mg/L of dicamba], and II: auxin and cytokinin (1.5 mg/L of 2,4-D + 0.5 mg/L of kinetin). The results showed that the spring genotypes were higher responsive considering the embryo-like structures and green plantlets in C17 with hormone I and C17 with hormone II compared with the winter ones. Besides, within the spring wheat genotypes, higher androgenetic production of embryo-like structures and green plantlets

was obtained in C17 with hormone I compared with C17 with hormone II, on the contrary of that, the winter wheat generated a higher frequency of embryo-like structures and green plantlets in C17 with hormone II compared with C17 with hormone I, thus the selection of the appropriate composition of the medium is crucial for increasing the effectiveness in anther culture.

Caffeine or trifluralin was used at the beginning of the induction phase to improve the early doubling of chromosomes and androgenetic induction (BROUGHTON et al. 2020). Caffeine can stimulate the formation of embryo-like structures and the regeneration of green plantlets and affects the phragmoplast microtubules during cell division and cytokinesis (YASUHARA 2005). This was demonstrated in the study by BROUGHTON et al. (2020), which reported the occurrence of modest improvements in the regeneration of green plantlets in two crosses of six spring wheat when applying a 0.5 mM caffeine treatment for 24 h at the beginning of the induction phase to improve androgenesis induction and early genome doubling. The increase in green plantlets was 14% in one cross and 27% in the other. Besides, the rearrangements of cytoskeleton reprogramme microspores toward androgenesis after the stress pre-treatment (TOURAEV et al. 2001; SEGUÍ-SIMARRO and NUEZ 2008).

Colchicine and many herbicides disturb spindle microtubules and have prompted the microspore embryogenesis in various species, while *n*-butanol influences cortical microtubules and has prompted the androgenesis in wheat (SORIANO et al. 2008; BROUGHTON 2011).

DING et al. (1991) showed that a low dose of gamma-ray (up to 7 Gy) could improve anther culture response in wheat as well.

3.3.5.3. Composition of anther culture media and culture conditions

Many studies have been performed in recent decades to improve the efficiency of anther culture induction medium. The most frequently applied induction media for androgenesis in anther culture of winter wheat are P_4 (PAUK et al. 2003), W_{14} (RUBTSOVA et al. 2013; LANTOS et al. 2013; LANTOS and PAUK 2016; LAZARIDOU et al. 2016), and P_2 (KONDIC-SPIKA et al. 2011). There are other induction media, too, such as C17 (WEIGT et al. 2020), LIM (BROUGHTON et al. 2020), MS3M (SORIANO et al. 2007; SANCHEZ-DIAZ et al. 2013; CASTILLO et al. 2015) and AM (REDHA et al. 2000; REDHA and SULEMAN 2011). These media contain maltose as a carbon source (HUNTER 1987) and ficoll as an osmotic agent (DATTA and WENZEL 1987). Recently, W14 and MS3M media have been widely applied in haploid experiments and wheat breeding programmes. W14 medium has been modified to W14mf synthetic medium, which has been efficiently applied in our wheat research and breeding programmes (LANTOS et al. 2013, LANTOS and PAUK 2016; KANBAR et al. 2020).

Some organic components, such as potato extract and wheat ovaries were reported to increase the efficiency of *in vitro* anther culture (DATTA and WENZEL 1987; BROUGHTON 2008, 2011; CASTILLO et al. 2015; BROUGHTON et al. 2020).

The most frequently used regeneration media are 190-2 (TUVESSON et al. 2000; PAUK et al. 2003; LANTOS et al. 2013; LANTOS and PAUK 2016; LAZARIDOU et al. 2016; ORLOWSKA et al. 2020; KANBAR et al. 2020), J25-8 (SORIANO et al. 2007, 2008; CASTILLO et al. 2015) and MS (RUBTSOVA et al. 2013; WEIGT et al. 2016, 2019). The embryo-like structures require an incubation period of approximately two weeks at 22–26°C with 16 h photoperiod in a growth chamber to regenerate green and albino plantlets in a different ratio.

3.3.6. Green plantlet production via *in vitro* anther culture

Although many studies achieved progress in *in vitro* anther culture improvements by following a specific protocol (BROUGHTON et al. 2008, 2011, 2020; SORIANO et al. 2008; LANTOS et al. 2013), there was still a variation between wheat genotypes in response to anther culture method. Some studies reported a maximum green plantlet production higher than 100 green plantlets/100 anthers (BROUGHTON 2011, 2020; LANTOS et al. 2013; CASTILLO et al. 2015). Other researchers reported maximum values less than 25 green plantlets/100 anthers (KIM and BAENZIGER 2005; KHIABANI et al. 2008; KONDIC-SPIKA et al. 2008; EL-HENNAWY et al. 2011; GRAUDA et al. 2014; WEIGT et al. 2019; ORLOWSKA et al. 2020; KANBAR et al. 2020). In some publications, the recorded maximum values were between 25–37 green plantlets/100 anthers (TROTTIER et al. 1993; NAVARRO-ALVAREZ et al. 1994; LANTOS et al. 2013; WEIGT et al. 2020). The overall mean of green plantlet production/100 anthers ranging between 0.40 and 9.76 green plantlets/100 anthers depending on the applied protocol was recorded from several previous winter wheat breeding programmes of MASOJC et al. (1993); HOLME et al. (1999); TUVESSON et al. (2000); KONDIC-SPIKA et al. (2008); EL-HENNAWY et al. (2011); GRAUDA et al. (2014); WEIGT et al. (2019, 2020); KANBAR et al. (2020).

3.3.7. Chromosome doubling

The haploid plants regenerated from diploid species have only one set of chromosomes and are characterized by being smaller, weak, and infertile because chromosomes cannot pair during meiosis. They could spontaneously restore their fertility or stimulants are needed for achieving artificially-induced diploidization. The chromosome doubling occurs from the application of any factor that prevents spindle formation during mitosis and thus hindering the normal segregation of sister chromatids toward the poles. The doubled haploid plants are homozygous at all loci representing a new genotype.

Spontaneously doubled haploid is commonly shown among cereal plants produced by anther culture. It is a safe process because the colchicine has a toxic effect to humans (DHOOGHE et al. 2011). Spontaneous chromosome doubling, which restores the fertility in cereals, provides the chance to avoid the examination of regenerated plants for ploidy determination, the treatment of haploid plants with colchicine by root immersion (JENSEN 1974; INAGAKI 2003), and also to avoid the problems of plants associated with mortality, ploidy chimaeras and variable seed set caused by this treatment (SORIANO et al. 2007). Nuclear fusion is widely-known as a mechanism for spontaneous chromosome doubling in microspore-derived haploid wheat and barley (KASHA 2005; DAGHMA et al. 2014). However, for winter wheat, the spontaneous doubling rate varied between 25% and 70% in the report of CASTILLO et al. (2009). LANTOS and PAUK (2016) recorded from 17.65% to 60%. WEIGT et al. (2019) obtained spontaneous doubled haploid plant rate ranging between 27% and 43% depending on the genotype. BROUGHTON et al. (2020), who treated Australian spring wheat crosses with caffeine or trifluralin, achieved from 14% to 80% spontaneous rates. Overall rates of spontaneous doubled haploid were 49%, 47.90%, 35%, and 32.72% in the researches of KIM and BAENZIGER (2005), KONDIC-SPIKA et al. (2008), LANTOS et al. (2013), and LANTOS and PAUK (2016), respectively. Spontaneous doubled haploid winter wheat lines varying between 5% and 30% were found in early studies conducted by ZIEGLER et al. (1990), MASOJC et al. (1993), and NAVARRO-ALVAREZ et al. (1994).

Colchicine has been successfully added to anther and microspore culture media at an early stage, to improve genome doubling in wheat (SORIANO et al. 2007; BARNABÁS et al. 1991; HANSEN and ANDERSEN 1998a). Colchicine should be used at relatively high concentrations to achieve affinity to plant microtubules and thus the chromosome doubling (MOREJOHN et al. 1984; MOREJOHN et al. 1987a), but the negative aspect of this application is that colchicine has a toxic effect on humans and a high affinity to vertebrate microtubules (DHOOGHE et al. 2011). The 0.1% (w/v) (2.5 mM) concentration of colchicine is commonly used for root immersion treatment in cereals (JENSEN 1974; INAGAKI 2003), however, lower concentrations between 0.3 and 1.0 mM are used in *in vitro* anther and microspore cultures (SORIANO et al. 2007; HANSEN and ANDERSEN 1998a). In the study of SORIANO et al. (2007), they observed that the wheat variety Paven achieved smaller improvements in a chromosomal doubling when using colchicine in anther culture compared with microspore culture.

Many herbicides target mitosis and have a mechanism for doubling the chromosomes such as dinitroanilines (trifluralin and oryzalin), benzamides (pronamide), phosphoro-thioamidates (aminoprophos-methyl or APM), and carbamates (chlorpropham and isopropyl N-3-chlorophenyl carbamate) (DHOOGHE et al. 2011). Studies have illustrated the mechanism of oryzalin and APM, which covers binding to tubulin proteins, inhibiting the polymerization of microtubules and

stimulating the depolymerization of the anaphase spindle (MOREJOHN et al. 1987b; MURTHY et al. 1994). Mitosis and cell division are prohibited; also, affected cells may include polyploid nuclei. These chemicals have induced diploidisation in several plant species (DHOOGHE et al. 2011). In addition to colchicine, trifluralin, oryzalin, and APM have also been applied for chromosome doubling during androgenesis in wheat (HANSEN and ANDERSEN 1998b). Since these chemicals have a much higher affinity to plant microtubules than colchicine, thus they can be used in micromolar concentrations (MOREJOHN et al. 1987b; BAJER and MOLÈ-BAJER 1986). Furthermore, these chemicals do not bind to animal microtubules (MOREJOHN et al. 1987b; MURTHY et al. 1994; BAJER and MOLÈ-BAJER 1986), thereby reducing the risk of toxicity to humans. In the previous studies, relatively higher concentrations of oryzalin, trifluralin and APM have had the similar *in vitro* effects to colchicine in improving the chromosome doubling in wheat. The highest rate of fertile plants has been obtained by the concentration of 10 μM trifluralin or APM applied for 48 h.

In anther culture, androgenesis and early genome doubling can be obtained if chemical herbicides, such as caffeine or trifluralin are applied at an early stage in the induction medium needed for embryo-like structure stimulation, then doubled haploid and fertile plants are spontaneously produced (BROUGHTON et al. 2020). The study conducted by BROUGHTON et al. (2020) revealed that trifluralin had a significant improvement in the chromosome doubling in the control genotype of wheat after pre-treatment of 1 μM and 3 μM for 48 h from 38% to 51% and 53%, respectively. Trifluralin, however, resulted in a negative effect on the green plantlet regeneration per 20 anthers concerning the same genotype and reduced the number from 31.8 to 9-25. Use of caffeine in this experiment did not achieve significant improvements in chromosome doubling in wheat anther culture, while it was not tested for *in vitro* microspore culture as an agent for the same purpose. Caffeine has been tested in haploid interspecific (wheat × maize) crosses instead of colchicine to double the genome in wheat (THOMAS et al. 1997). In that study, root immersion treatments with tested concentrations of 0.3–10 g/L and time of 3–24 h have been applied. Caffeine can be used as an agent for restoring fertility in wheat through immersion/root dipping treatment and the best result of the discovered fertility could be obtained after applying 3 g/L (15.4 mM) for 24 h under different tested concentrations (THOMAS et al. 1997).

Sugar starvation was widely-used as stress pre-treatment including putting the anthers on a medium containing mannitol as a carbohydrate source (CAREDDA et al. 2000; KASHA et al. 2001; CISTUÉ et al. 2006; SORIANO et al. 2007; CASTILLO et al. 2015). This pre-treatment led to high rates of chromosome doubling in barley (KASHA et al. 2001; SHIM et al. 2006), and wheat (HU and KASHA 1997).

4. MATERIALS AND METHODS

4.1. Characterization of winter wheat genotypes for drought tolerance

4.1.1. Plant material and cultivation method

This study involved nine wheat genotypes: six pre-selected doubled haploid lines originating from a mapping population for drought tolerance at Cereal Research Non-profit Ltd., Szeged, Hungary, and divided into two groups based on the study of NAGY (2019) – drought-tolerant (PC61, PC110, and PC332) and drought-sensitive (PC84, PC92, and PC94) – and three other varieties from different sources. The latter involved varieties: 'Plainsman V.' (drought-tolerant), 'GK Berény' (drought-tolerant), and 'GK Élet' (drought-sensitive) and were used as control genotypes under well-watered and drought stress conditions. 'Plainsman V.' is a drought-tolerant variety developed in Kansas, USA, in 1974. It is a hard red winter wheat, which provides moderate grain yield with high protein content, and matures early. 'GK Berény' is a drought-tolerant and early maturing variety registered in Hungary. 'GK Élet' is also a Hungarian early maturing variety. As for the doubled haploid lines, they were originated from the cross between the drought-tolerant 'Plainsman V.' and the French drought-sensitive variety 'Capelle Desprez' (GALLÉ et al. 2009). They were developed through anther culture from the F_1 generation according to the protocol of PAUK et al. (2003). The first phenotyping experiment was conducted in the 2017–2018 season (NAGY 2019) in the glasshouse of the Cereal Research Non-profit Ltd. in Szeged. The grains were sown on a 1:1 soil and sand mixture in a growing chamber.

Figure 4. Transplanting seedlings into plastic pots filled with a soil mixture.

One-week-old seedlings were transferred to a cold chamber for vernalisation for six weeks at 4°C under constant dim light. After the vernalization, each seedling was transplanted into a plastic pot (Figure 4) filled with a soil mixture of 520 g peat soil, 1276 g dry sandy soil, and 3 g controlled-release fertilizer (Osmocote® Exact®, Scotts® Company, Marysville, Ohio) involving NPK (16%, 9%, 12% respectively), MgO 2.5% and microelements.

4.1.2. Water management

Before planting, the water capacity of the soil mixture used was estimated by calculating the difference between the weight of the air-dry soil and the water-saturated soil (CSERI et al. 2013). 100 mL of water was then supplied to each seedling to ensure adaptation. Each genotype per treatment was given the same amount of water each time (twice a week) with the average irrigation requirement of the plants, which varied each irrigation day. The average irrigation requirement was determined for each of the plants by calculating the difference between the mean value of five well-watered pots weight and the control weight (the difference between the weight of air-dry soil and water-saturated soil). The plants of well-watered treatment were irrigated to 60% soil water capacity, while the plants of drought stress treatment were irrigated to one-third of the soil water capacity. The total amount of water applied to each plant during the growing season was 4962 mL in the well-watered treatment, and 1654 mL in the drought stress treatment.

4.1.3. Investigated traits

Several morphological traits were recorded, such as days to heading calculated for each plant when the upper half of the main spike emerged from the flag leaf sheath. Plant height was measured from the ground to the top of the spike, not including the length of awn, after flowering (Figure 5).

When grains matured, the plants were harvested as a whole, and each plant was put into a thermostat cabinet in a paper box for drying at 42°C until the weight became stable. A group of traits were then recorded involving above-ground biomass weight, main spike length, spikelet number/plant, fertile spikelet number/plant, grain number/plant, grain yield/plant, harvest index, 1000-grain weight, root length, and root dry mass.

Two weeks after harvesting, the roots were carefully removed from the soil and washed (Figure 6), before being dried at 27–32°C for 2 weeks in the shade, after which the root dry mass was estimated.

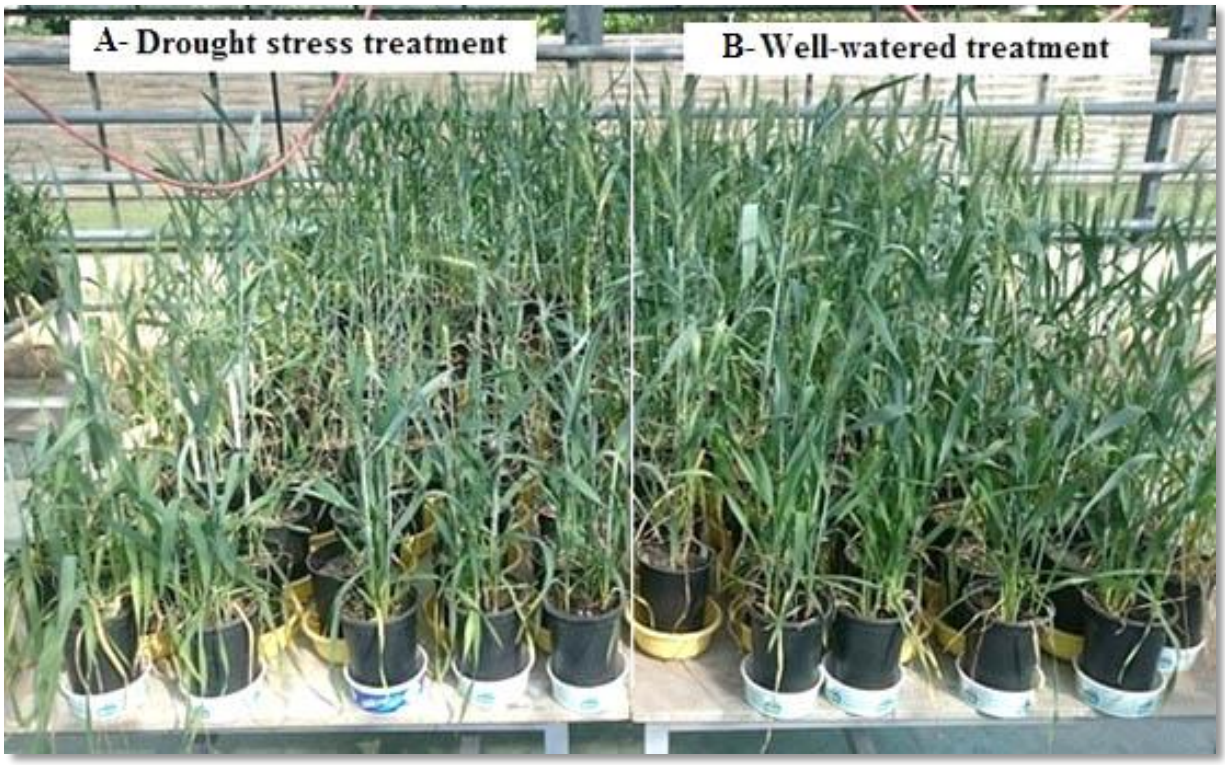

Figure 5. The investigated winter wheat genotypes at heading stage under drought stress (A) and well-watered treatments (B).

Figure 6. Washing the roots removed from the soil after two weeks of harvest, before being dried in the shade.

4.1.4. Experimental design and statistical analysis

The experiment was conducted in a randomized complete block design with well-watered and drought stress treatments and five replications (Figure 7), and lasted from 31st January 2019 to 10th July 2019, where the standard glasshouse wheat-growing programme was applied according to CSERI et al. (2013) and PAUL et al. (2016).

The recorded data were inserted into an Excel programme and analysed using R software (Ver. 3.6.1., R CORE TEAM 2019). Two-way ANOVA was used to calculate the coefficient of variation (CV), standard errors (SE), the least significant differences ($LSD_{0.05}$), sums of squares (SS), mean squares (MS), the interaction between genotypes and treatments, F values, and F probabilities for all the tested traits. The correlation matrix was generated using Pearson product-moment correlation and pairwise-P values to determine the significance of correlation coefficient values. The fitted linear regression model was used to examine the relationship between the traits. For each trait, comparative analysis between well-watered and drought stress treatments was performed to calculate the reduction value and the percent reduction. Stress tolerance index (STI) was calculated according to FERNANDEZ (1992), where $STI= (y_w + y_s)/\bar{y}^2_w$, y_w is the grain yield/plant of a genotype under well-watered treatment, y_s is the grain yield/plant of a genotype under drought stress treatment, and $\bar{y}_w$ is the mean of grain yields/plant of all studied genotypes under well-watered treatment.

Figure 7. The experimental design (9 wheat genotypes × 2 treatments × 5 replications).

4.2. Generation of winter wheat doubled haploid lines via *in vitro* anther culture

4.2.1. Plant materials

Thirteen F_4 combinations (accessions) were selected for this study from the drought tolerance trial of thirteen winter wheat F_3 plant materials (Table 1) provided by the Cereal Research Non-profit Ltd. (CR Ltd.). The grains were sown on 5 m^2 plots/combination (450 grain/m^2) at the CR Ltd. in October 2017. The agricultural practices of the wheat crop were applied from fertilization to pest control depending on the standard protocol for small grain winter cereals. The required fertilizers (N:P:K = 1:1:1) were added in autumn, and the ammonium nitrate was applied in mid-April 2017 at a dose of 18 g/m^2. The insect pest protection was carried out by the application of Bulldock® (Bayer Crop Science, Budapest, Hungary) as required. Besides, weed control was performed by using the herbicide Pointer star® (DuPont Mo. Ltd., Budaörs, Hungary) accompanied by mechanical methods during the growing season.

Table 1. List of the wheat F_4 combinations tested in the anther culture
[The crossing combinations were selected in the previous (F_3) generation based on the yield performance and drought tolerance under different ecological conditions]

No	Code number	Combinations
1	2522	Sel.9/DH150
2	2533	Premio/5009
3	2570	DL41/DH150
4	2572	DL45/DH150
5	2581	Béres/Midas
6	2591	Béres/Pamier
7	2610	Kalász/Tacitus
8	2635	Kolo/Premio
9	2680	Körös/Premio
10	2712	Midas/Csillag//Tacitus/5003
11	2739	DH54/12.189
12	2740	DH54/12.89
13	2744	Kapos/Ködmön

4.2.2. Collection and treatment of donor tillers

About 35–40 donor tillers (containing microspores at the early-uninucleate stage) of each tested genotype were collected from 25th April to 3rd May 2018 (Figure 8A), placed in Erlenmeyer flasks with tap water, covered with PVC bags and kept at 3–4^{o}C under continuous dim (200 µmol/m^2/s) fluorescent light for a 2-week cold pre-treatment (Figure 8B).

4.2.3. Isolation and incubation of anthers

The selected cold pre-treated spikes, with microspores at the optimal developmental stage (checked under an Olympus CK-2 inverted microscope (Olympus, Southern-on-Sea, UK), Figure 8A) were sterilised under a flow box, placed in 250 mL Erlenmeyer flasks (containing 200 mL of 2% NaOCl solution (w/v) with one drop of Tween-80), covered and placed on a gyratory shaker for 20 min (120 RPM). The spikes were then rinsed three times in sterile distilled water (Millipore Elix 5). 300 anthers per replication were isolated using fine forceps and put onto a 90 mm plastic Petri dish (Sarstedt, Budapest, Hungary) containing 15 mL of a liquid W14mf induction medium (Table 2, Figure 8C). After the heat-shock treatment at 32°C for 36 h in the dark, the cultures were incubated at 28°C in the dark for about 5–8 weeks for embryo-like structure induction. 30–35 cold-pre-treated spikes were used for preparing 10 replications per genotype, with 300 anthers each.

4.2.4. Plantlet regeneration

About 5-weeks after the incubation, approximately 30–35 embryo-like structures with a diameter of 1–2 mm (Figure 8D) were transferred onto 30 mL Petri dishes filled with a 190-2Cu regeneration medium solidified with 2.8 g/L Gelrite® (PAUK et al. 2003, Table 2) and put in a lighted growth room. Transfer of the embryo-like structures lasted for about 8 weeks.

After about 2–3 weeks, approximately 15–18 of the green plantlets with a length of 20–30 mm (Figure 8E), were transferred into 1000 mL plastic boxes filled with a solid regeneration medium. In addition, the individual green plantlets were transferred into 50 mL glass tubes containing the same medium. The boxes and tubes were kept in a growth room (24°C, 16/8 h light/dark photoperiod, fluorescent light at 200 µmol/m^2/s) for the regeneration of whole plantlets (Figure 8G). The albino plantlets were counted and thrown away (Figure 8F).

4.2.5. Acclimatization of plantlets and harvest of doubled haploid grains

About 4–5 weeks later, the well-rooted plantlets were transferred to the glasshouse and transplanted into plastic pots (Figure 8H) containing a mixture of peat and sand (1:1). The plantlets were covered with a PVC, and initially kept at 17–22°C for 3–5 days for the acclimatisation. After about 2–3 weeks, the plants were moved to a cool chamber (8–12°C under 16/8 h light/dark) for additional 2–3 months before transplantation to the nursery.

In October, the plants in the cold chamber were transplanted to the nursery (Figure 8I). During the growing season in autumn and winter, many different stresses (cold, frost, short days etc.) affect the plants restoring the fertility. The number of double haploid plants depends on the applied stresses. At the end of the growing season, all of the partially fertile and fertile spikes were

manually harvested; the plants with entire sterile spikes were counted and discarded (Figure 9). For data analysis, the doubled haploid plants were divided into two groups depending on the type of spike fertility: fully fertile with 100% and partially fertile with less than 100% seed set.

Table 2. Composition of the media used in wheat anther culture

Media components	Induction medium W14mf (mg/L)	Regeneration medium 190-2CU (mg/L)
Macro salts		
KNO_3	2,000	1,000
KCl	-	40
K_2SO_4	700	-
(NH4)2SO4	-	200
KH2PO4	-	300
$NH_4H_2PO_4$	380	-
$CaCl_2 \cdot 2H_2O$	140	-
Ca(NO3)2 · 4H2O	-	100
$MgSO_4 \cdot 7H_2O$	200	200
Iron source		
Na_2EDTA	37.3	37.3
$FeSO_4 \cdot 7H_2O$	27.8	27.8
Micro salts		
$MnSO_4 \cdot 4H_2O$	8	8
$ZnSO_4 \cdot 7H_2O$	3	3
H_3BO_3	3	3
KI	0.5	0.5
$CuSO_4 \cdot 5H_2O$	0.025	0.5
$CoCl_2 \cdot 6H_2O$	0.025	-
$Na_2MoO_4 \cdot 2H_2O$	0.005	-
Vitamins		
Myo-Inositol	-	100
Thiamine HCl	2	1
Pyridoxine HCl	0. 05	0.5
Nicotinic acid	0. 05	0.5
Other components		
Glycine	-	2
Sucrose	-	30,000
Maltose	90,000	-
2,4-D	2	-
Kinetin	0.5	0.5
NAA	-	0.5
Ficoll 400	100,000	-
Gelrite	-	3,000
pH	5.8	5.8

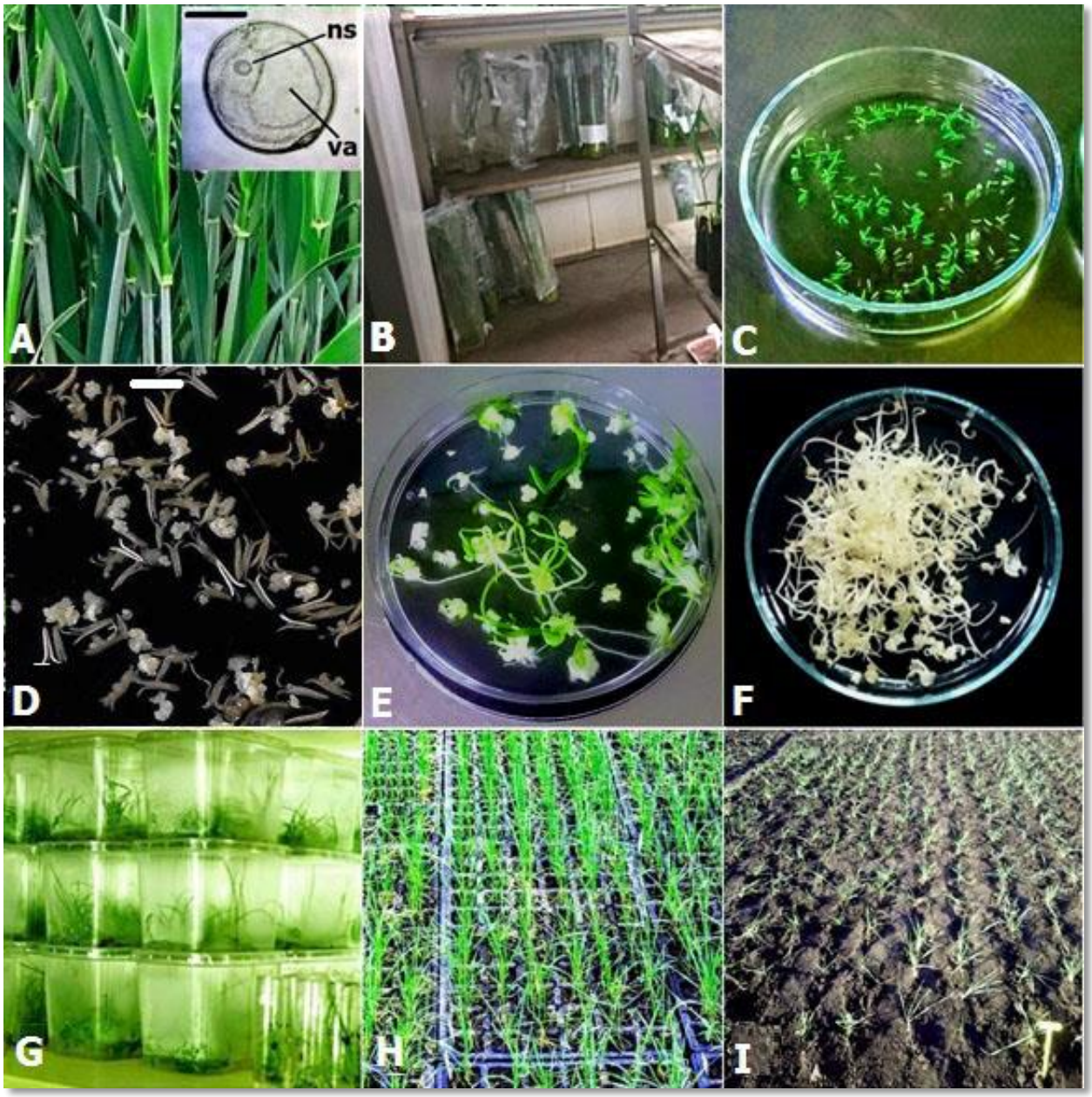

Figure 8. Main stages of the wheat anther culture: donor tillers in the nursery when the microspores are in the uninucleate developmental stage (right upper corner of A; ns – nucleus, va – vacuole) (A); cold pre-treatment of wheat tillers for 2 weeks at 3–4°C under continuous dim light in a cold chamber (B);isolated anthers on the surface of the W14mf liquid medium (C); embryo-like structures obtained in the four-week-old anther culture (D); green plantlets on the regeneration medium (E); collected and discarded albino plantlets from the plant regeneration (F); well-rooted green plantlets in plastic boxes (G); transplanted plantlets in the glasshouse (H); transplanted plantlets in the field (I). Bar = 10 µm (A) or 4 mm (D).

Figure 9. Wheat doubled haploid sterile spikes.

4.2.6. Statistical analysis

The anther culture experiment comprised 10 replications per genotype and 300 anthers/replication. The effect of the genotype was tested, and the collected data of the androgenetic parameters (number of embryo-like structures, regenerated-, green-, albino-, and transplanted plantlets) were analysed using the ANOVA (analysis of variance) of the R software (Ver. 3.6.1., R CORE TEAM, 2019). The pairwise comparisons of the means were computed as well.

5. RESULTS

5.1. Characterization of winter wheat genotypes for drought tolerance

5.1.1. The response of the studied traits to water deficit

The statistical analysis of variance (two-way ANOVA) for all the investigated traits is demonstrated in Table 3. High significant differences of genotype and treatment effects were recorded in all traits except root length. For root length, the genotype effect was significant at $P < 0.01$ probability level, while the treatment effect was significant at $P < 0.05$ probability level.

Table 3. Analysis of two-way ANOVA for each studied trait
[(*), (**), (***) significant differences at the 0.05, 0.01, 0.001 probability levels, respectively, (DF) degrees of freedom, (SS) sum of squares, (MS) mean square, (Pr) probability, (CV) coefficient of variation, (LSD) least significant difference]

Traits	Resource of variance	DF	SS	MS	F value	Pr (>F)
Heading time (day)	Genotype	8	1839.20	229.9	134.36	0.000***
CV=7.74%	Treatment	1	214.68	214.68	125.46	0.000***
LSD=1.91	Genotype * Treatment	8	79.82	9.98	5.83	0.000***
	Error	72	123.20	1.71		
Plant height (cm)	Genotype	8	3197.4	399.68	31.049	0.000***
CV= 21.27%	Treatment	1	10070	10070	782.308	0.000***
LSD= 5.23	Genotype * Treatment	8	616.4	77.05	5.985	0.000***
	Error	72	926.8	12.87		
Above-ground	Genotype	8	89.26	11.16	16.3998	0.000***
biomass (g)	Treatment	1	1351.48	1351.48	1986.387	0.000***
CV= 57.18%	Genotype * Treatment	8	14.24	1.78	2.616	0.014*
LSD= 1.20	Error	72	48.99	0.68		
Main spike length (cm)	Genotype	8	106.316	13.29	70.191	0.000***
CV= 17.65%	Treatment	1	91.405	91.41	482.775	0.000***
LSD=0.63	Genotype * Treatment	8	4.916	0.61	3.245	0.003**
	Error	72	13.632	0.189		
Spikelet number/plant	Genotype	8	8823	1102.88	11.355	0.000***
CV= 43.38%	Treatment	1	32642	32642	336.07	0.000***
LSD= 14.37	Genotype * Treatment	8	1465	183.13	1.885	0.075
	Error	72	6993	97.125		
Fertile spikelet number/plant	Genotype	8	7880	985	16.84	0.000***
CV= 55.80%	Treatment	1	39313	39313	672.14	0.000***
LSD= 11.15	Genotype * Treatment	8	882	110.25	1.885	0.075
	Error	72	4211	58.486		
Grain number/plant	Genotype	8	38132	4766.5	13.478	0.000***
CV= 61.50%	Treatment	1	325442	325442	920.21	0.000***
LSD= 27.43	Genotype * Treatment	8	7380	922.5	2.608	0.014*
	Error	72	25464	353.67		
Grain yield/plant (g)	Genotype	8	27.68	3.46	10.43	0.000***
CV= 67.82%	Treatment	1	424.71	424.71	1280.7	0.000***
LSD= 0.84	Genotype * Treatment	8	4.48	0.56	1.688	0.0116
	Error	72	23.88	0.332		
Harvest Index %	Genotype	8	1754.5	219.31	8.669	0.000***
CV= 21.14%	Treatment	1	3719.7	3719.7	147.03	0.000***
LSD= 7.34	Genotype * Treatment	8	443.9	55.488	2.193	0.037*
	Error	72	1821.5	25.299		
1000-grain weight (g)	Genotype	8	1659.65	207.46	16.366	0.000***
CV= 21.47%	Treatment	1	924.71	924.71	72.95	0.000***
LSD= 5.19	Genotype * Treatment	8	296.11	37.014	2.92	0.007**
	Error	72	912.68	12.676		
Root length (cm)	Genotype	8	492.6	61.58	2.932	0.0068**
CV= 21.62%	Treatment	1	96.1	96.1	4.576	0.0358*
LSD= 6.68	Genotype * Treatment	8	368.2	46.025	2.192	0.0379*
	Error	72	1512	21		
Root dry mass (g)	Genotype	8	0.462	0.058	13.255	0.000***
CV= 55.07%	Treatment	1	0.375	0.375	86.024	0.000***
LSD= 0.09	Genotype * Treatment	8	0.082	0.01	2.337	0.027*
	Error	72	0.314	0.004		

The results of genotype and treatment interaction effect showed that significant differences at $P < 0.001$ probability level were obtained in the heading time and plant height traits, and at $P < 0.01$ probability level in the main spike length and 1000-grain weight traits, while significant differences at $P < 0.05$ probability level were recorded in the traits of above-ground biomass, grain number/plant, harvest index, root length and root dry mass; by contrast, non-significant differences of genotype and treatment interaction were present in the spikelet number/plant, fertile spikelet number/plant and grain yield/plant.

In this investigation, the influence of water deficit on wheat genotypes was observed on all the studied traits, since the plants changed their phenotype and dry matter accumulation in response to drought stress. Figures 10–33 reveals the effect of drought stress on the tested traits.

5.1.1.1. Heading time

The number of days to heading varied between 60.2 days in 'GK Élet' and 76 days in 'Plainsman V.' under well-watered conditions, and between 58.2 days in 'GK Élet' and 76.40 days in 'Plainsman V.' under drought stress. Drought caused a reduction in days to heading in all genotypes, as compared to the well-watered conditions, except for 'Plainsman V.', for which the number of days to heading increased by 0.40 of a day under drought compared to the well-watered conditions. Values of the reduction due to drought were significant in all genotypes except 'Plainsman V.' and 'PC92'. The reduction was the highest in 'PC84' and 'PC110' (6.60 and 4.40 days, respectively) while the lowest decrease values were achieved in 'PC92', 'GK Élet' and 'PC332' genotypes (1.40, 2, and 2.60 days, respectively) (Figure 10, Table 4).

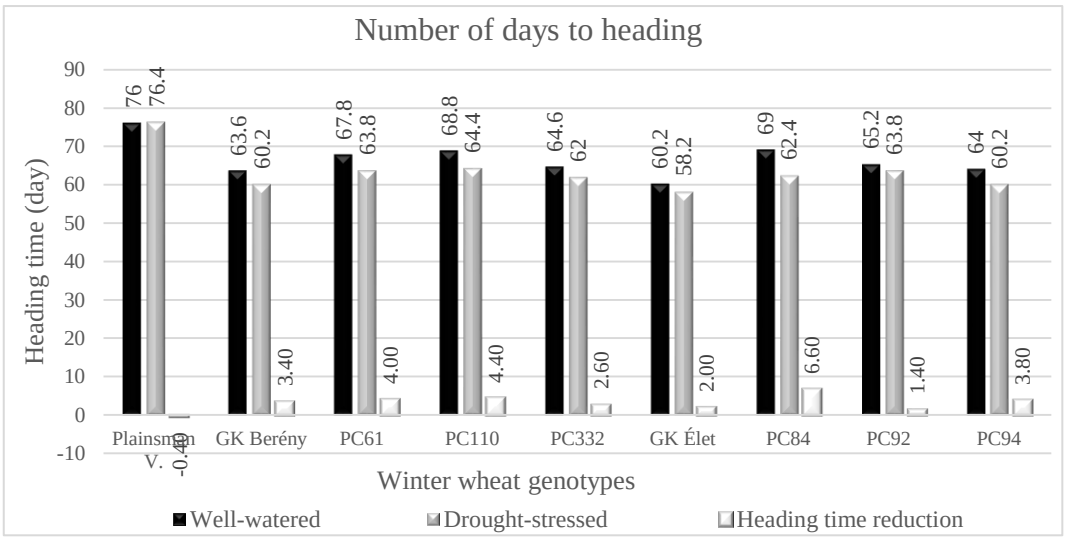

Figure 10. Heading time of nine wheat genotypes under well-watered and drought stress conditions.

Table 4. Mean of all studied traits and reduction (R) values for nine wheat genotypes under well-watered (WW) and drought stress (DS) conditions

No	Genotypes	Heading time (day)			Plant height (cm)			Above-ground biomass (g)			Main spike length (cm)			Spikelet number/plant			Fertile spikelet number/plant		
		WW	DS	R	WW	DS	R	WW	DS	R	WW	DS	R	WW	DS	R	WW	DS	R
1	Plainsman V.	76	76.4	-0.40	75.6	64.6	11	14	4.8	9.62	11.3	9.64	1.66	100	48.2	51.8	87.6	35.8	51.8
2	GK Berény	63.6	60.2	3.40	59.6	43.2	16.4	12	3.7	8.4	7.94	6.28	1.66	91	39.8	51.2	82	33.2	48.8
3	PC61	67.8	63.8	4.00	73.2	47.2	26	11	3.3	7.33	9.06	7.46	1.6	57.4	28.4	29	50.6	19.8	30.8
4	PC110	68.8	64.4	4.40	71.4	48	23.4	11	3.3	7.46	8.46	6.76	1.7	76.4	36	40.4	66.8	24.4	42.4
5	PC332	64.6	62	2.60	80.2	50.8	29.4	11	3	7.69	10.2	8.42	1.8	70.8	35.4	35.4	64.8	22	42.8
6	GK Élet	60.2	58.2	2.00	59	42.2	16.8	9.7	2.4	7.37	9.9	7.26	2.64	63.2	25.2	38	53.8	16.8	37
7	PC84	69	62.4	6.60	72	49.6	22.4	11	3.7	6.83	9.6	7.12	2.48	74.8	45.6	29.2	57.2	21	36.2
8	PC92	65.2	63.8	1.40	75.2	52.4	22.8	11	2.9	8	11.8	8.92	2.86	61.2	30.2	31	56.2	16.4	39.8
9	PC94	64	60.2	3.80	74.8	52.6	22.2	9.8	2.8	7.05	10.3	8.54	1.74	68	31.2	36.8	60	13.4	46.6

No	Genotypes	Grain number/plant			Grain yield/plant (g)			Harvest index (%)			1000-grain weight (g)			Root length (cm)			Root dry mass (g)		
		WW	DS	R	WW	DS	R	WW	DS	R	WW	DS	R	WW	DS	R	WW	DS	R
1	Plainsman V.	200.8	70.6	130.2	7.18	2.18	5	49.71	45	4.71	35.9	30.94	4.96	26	25.4	0.6	0.481	0.24	0.241
2	GK Berény	220.8	68	152.8	6.64	1.56	5.08	54.87	42.1	12.77	30.24	23.09	7.15	25.4	26	-0.6	0.339	0.184	0.155
3	PC61	162.2	52.6	109.6	5.5	1.22	4.28	51.69	37.29	14.4	33.82	24.28	9.54	19.6	27	-7.4	0.185	0.11	0.075
4	PC110	170.8	50.8	120	5.06	1.16	3.9	46.46	34.65	11.81	29.49	23.08	6.41	29	26.6	2.4	0.206	0.127	0.079
5	PC332	192	53	139	5.56	1.02	4.54	51.86	33.8	18.06	29.09	19.28	9.81	29.2	25.6	3.6	0.25	0.159	0.091
6	GK Élet	141	29.6	111.4	5.38	1.14	4.24	55.2	48.02	7.18	38.51	39.57	-1.06	18.2	26.2	-8	0.209	0.072	0.137
7	PC84	128.4	43.2	85.2	4.63	1.01	3.62	44.11	27.15	16.96	35.93	24.06	11.87	24.6	24.2	0.4	0.365	0.218	0.147
8	PC92	153.2	35.2	118	5.36	1.07	4.29	49.36	37.83	11.53	35.3	31.6	3.7	19.4	25	-5.6	0.3	0.105	0.195
9	PC94	148.6	32.4	116.2	5.09	0.93	4.16	51.55	33.23	18.32	34.2	28.91	5.29	18.6	22.6	-4	0.171	0.128	0.043

Figure 11 shows the reductions in heading time expressed as percentages of the values obtained under well-watered conditions. Eight genotypes showed an increase, while ‘Plainsman V.’ showed a decrease in these values under drought stress conditions. The percent reductions ranged from 2.15% in ‘PC92’ genotype to 9.57% in ‘PC84’ genotype; the lowest percent reductions of heading time trait were presented in the genotypes ‘PC92’, ‘GK Élet’ and ‘PC332’ (2.15, 3.32 and 4.02%, respectively), while the highest percent reductions were found in the genotypes ‘PC84’, ‘PC110’ and ‘PC94’ (9.57, 6.40 and 5.94%, respectively).

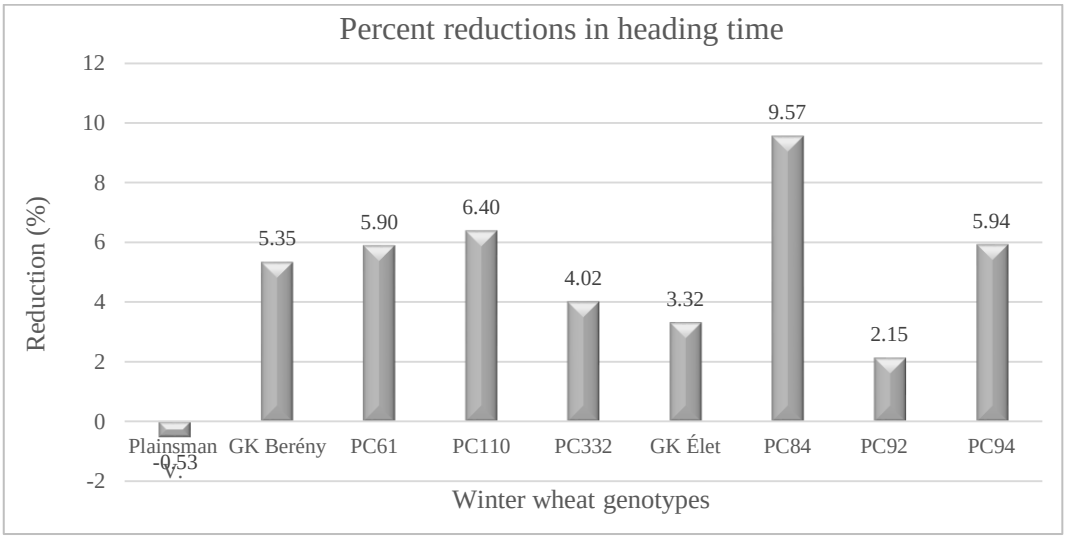

Figure 11. Percent reductions in heading time affected by water deficit.

5.1.1.2. Plant height

Water shortage significantly affected the plant height of each investigated genotype, as compared to the well-watered conditions. Plant height ranged between 64.60 cm in ‘Plainsman V.’ under drought stress and 75.60 cm in well-watered conditions, representing the smallest difference. ‘PC332’ had the highest difference, from 50.80 cm under drought stress to 80.20 cm in the well-watered conditions. The varieties ‘Plainsman V.’, ‘GK Berény’ and ‘GK Élet’ had the least decrease (11.00, 16.40 and 16.80 cm, respectively), while ‘PC332’ and ‘PC61’ showed the highest decrease in this trait: 29.40 and 26 cm, respectively (Figure 12, Table 4).

The percent reduction of plant height under drought stress conditions ranged between 14.56% in ‘Plainaman V.’ and 36.66% in ‘PC332’. The lowest plant height reduction rates were recorded in ‘Plainsman V.’, ‘GK Berény’ and ‘GK Élet’ (14.56, 27.52 and 28.47%, respectively), while the highest plant height reduction rates were obtained in the genotypes: ‘PC332’, ‘PC61’ and ‘PC110’ (36.66, 35.52 and 32.78%, respectively) (Figure 13).

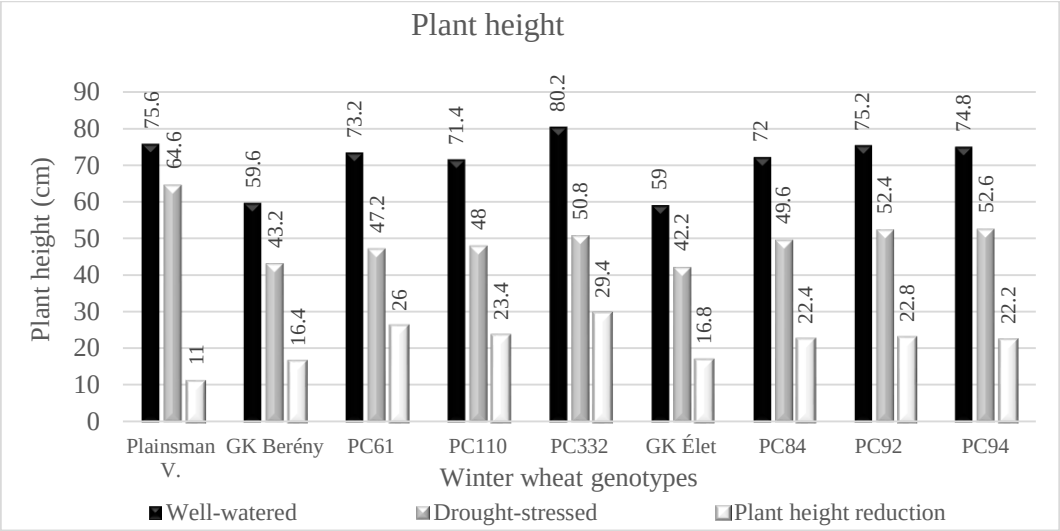

Figure 12. Plant height of nine wheat genotypes under well-watered and drought stress conditions.

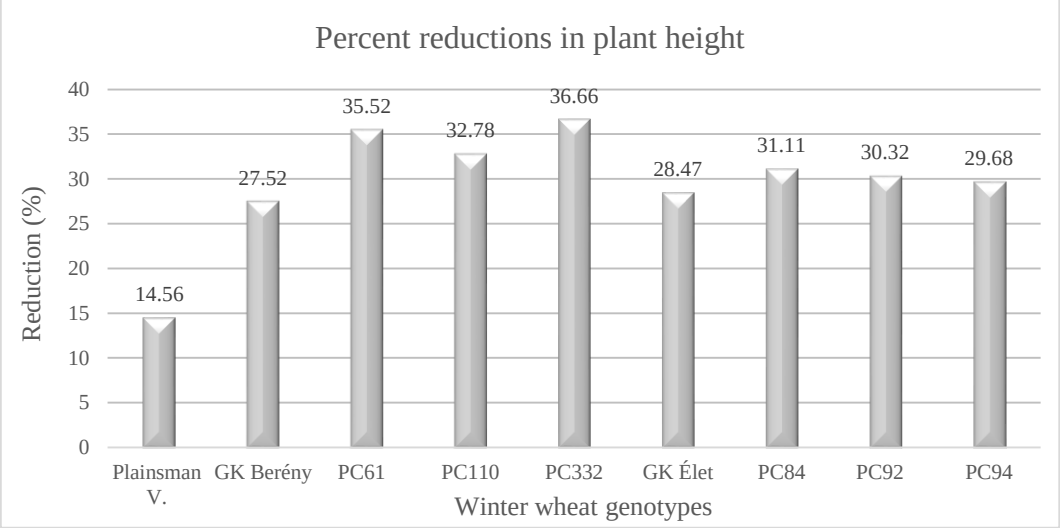

Figure 13. Percent reductions in plant height affected by water deficit.

5.1.1.3. Above-ground biomass

Each studied genotype exhibited a significant decrease in above-ground biomass when drought stress was applied compared to the well-watered conditions. The values of this trait varied between 9.73 g in ‘GK Élet’ and 14.46 g in ‘Plainsman V.’ in the well-watered conditions, and between 2.36 g in ‘GK Élet’ and 4.84 g in ‘Plainsman V.’ under water-stress treatment. The least decreases in above-ground biomass trait were found in the genotypes ‘PC84’, ‘PC94’ and ‘PC61’ (6.83, 7.05 and 7.33 g, respectively), while the highest decreases were observed at ‘Plainsman V.’, ‘GK Berény’ and ‘PC332’ (9.62, 8.40 and 7.69 g, respectively) (Figure 14, Table 4).

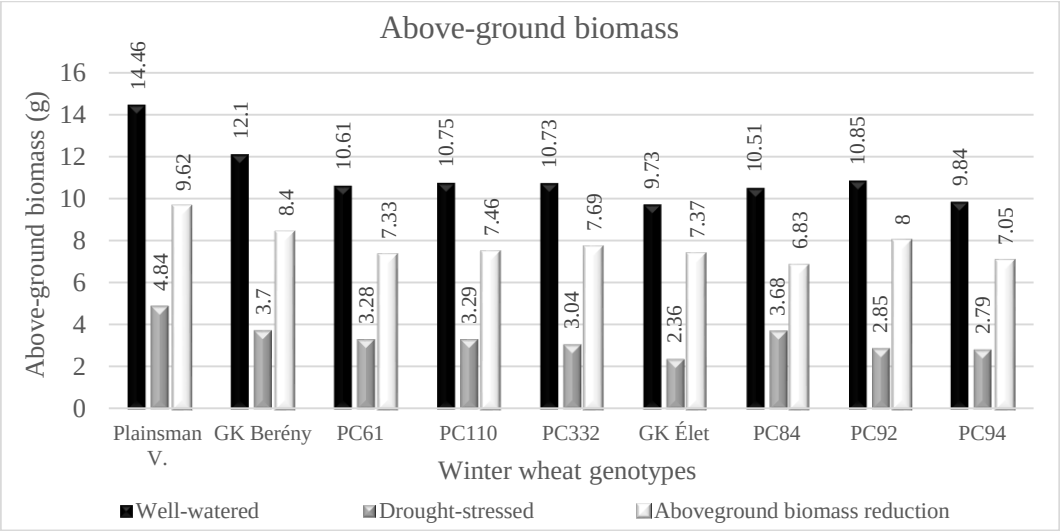

Figure 14. Above-ground biomass of nine wheat genotypes under well-watered and drought stress conditions.

The percent reduction of above-ground biomass caused by drought stress ranged from 64.99% to 75.75%, as compared to the well-watered conditions. The genotypes 'PC84' and 'Plainsman V.' achieved the lowest percent reduction (64.99 and 66.53%, respectively), while the percent reduction was the highest in 'GK Élet', 'PC92' and 'PC332' (75.75, 73.73 and 71.67%, respectively) (Figure 15).

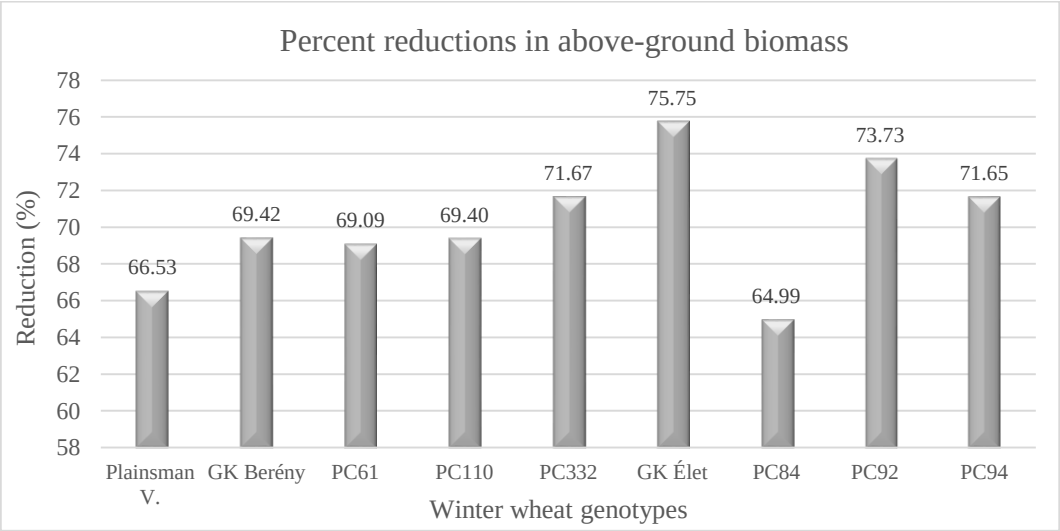

Figure 15. Percent reductions in above-ground biomass affected by water deficit.

5.1.1.4. Main spike length

A significant decrease in the main spike length was observed in each genotype under drought stress compared with well-watered conditions, ranging from 1.60 cm to 2.86 cm. The lowest decrease values were found in 'PC61', 'Plainsman V.' and 'GK Berény' (1.60, 1.66 and

1.66 cm, respectively), and the highest values of this decrease were recorded in ‘PC92’, ‘GK Élet’ and ‘PC84’ (2.86, 2.64 and 2.48 cm, respectively) (Figure 16, Table 4).

The percent reductions of this trait ranged between 14.69% and 26.67%. The wheat genotypes ‘Plainsman V.’, ‘PC94’ and ‘PC332’ achieved the lowest percent reduction values (14.69, 16.93 and 17.61%, respectively), while the genotypes ‘GK Élet’, ‘PC84’ and ‘PC92’ had the highest values of percent reduction (26.67, 25.83 and 24.28%, respectively) (Figure 17).

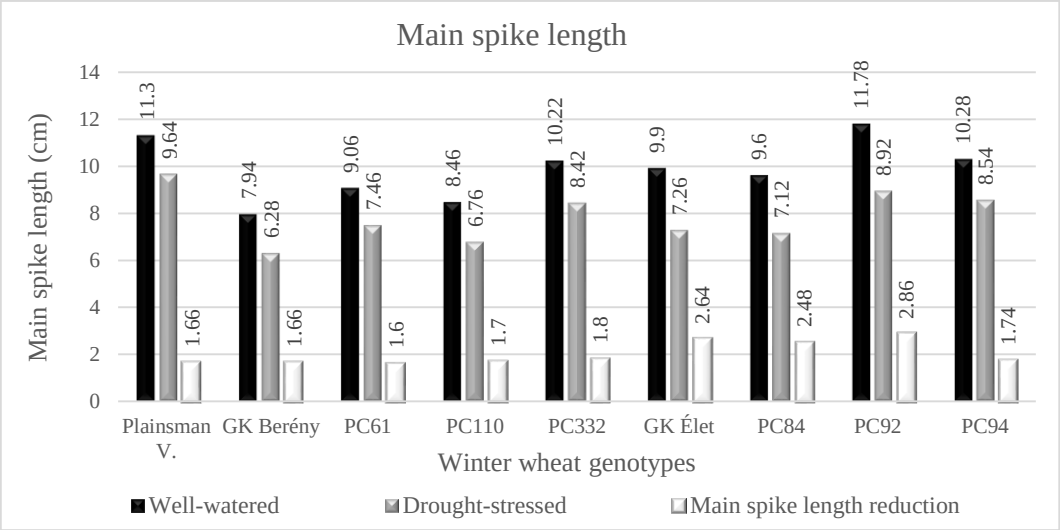

Figure 16. Main spike length of nine wheat genotypes under well-watered and drought stress conditions.

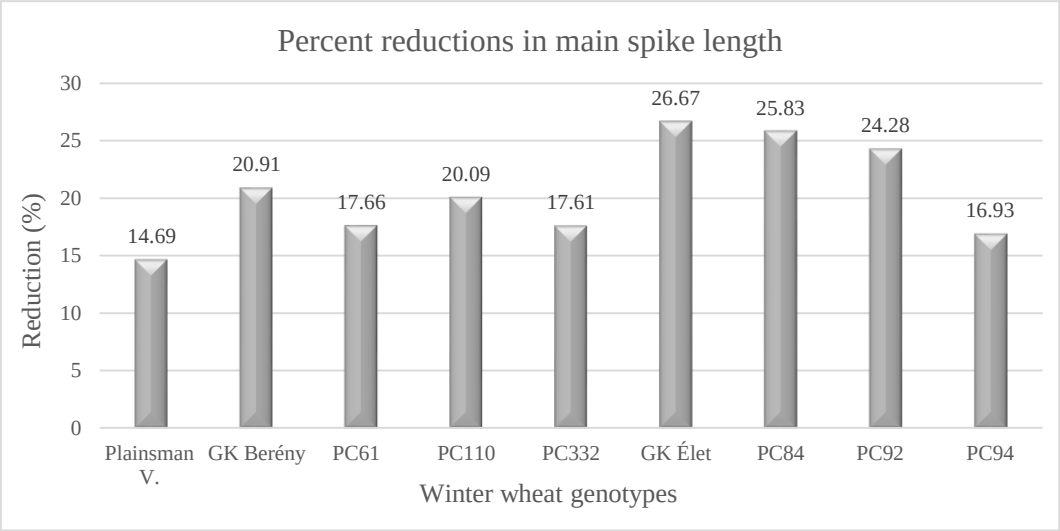

Figure 17. Percent reductions in main spike length affected by water deficit.

5.1.1.5. Spikelet number per plant

Drought conditions negatively affected the spikelet number per plant of each studied genotype compared with well-watered conditions. The values of spikelet number per plant varied from 57.40 in ‘PC61’ to 100 in ‘Plainsman V.’ under well-watered conditions and ranged from 25.20 spikelet number/plant in ‘GK Élet’ to 48.20 spikelet number/plant in ‘Plainsman V.’ under

water deficit conditions. The lowest reduction values belonged to ‘PC61’, ‘PC84’ and ‘PC92’ genotypes (29, 29.20 and 31 spikelet number/plant, respectively), while the highest reduction values were found in ‘Plainsman V.’, ‘GK Berény’ and ‘PC110’ (51.80, 51.20 and 40.40 spikelet number/plant, respectively) (Figure 18, Table 4).

The percent reduction of spikelet number/plant varied from 39.04% in ‘PC84’ to 60.13% in ‘GK Élet’. The lowest percent reduction values were present in ‘PC84’, ‘PC332’ and ‘PC61’ (39.04, 50 and 50.52%, respectively), while the highest values of percent reduction for this trait were recorded in ‘GK Élet’, ‘GK Berény’ and ‘PC94’ (60.13, 56.26 and 54.12%, respectively) (Figure 19).

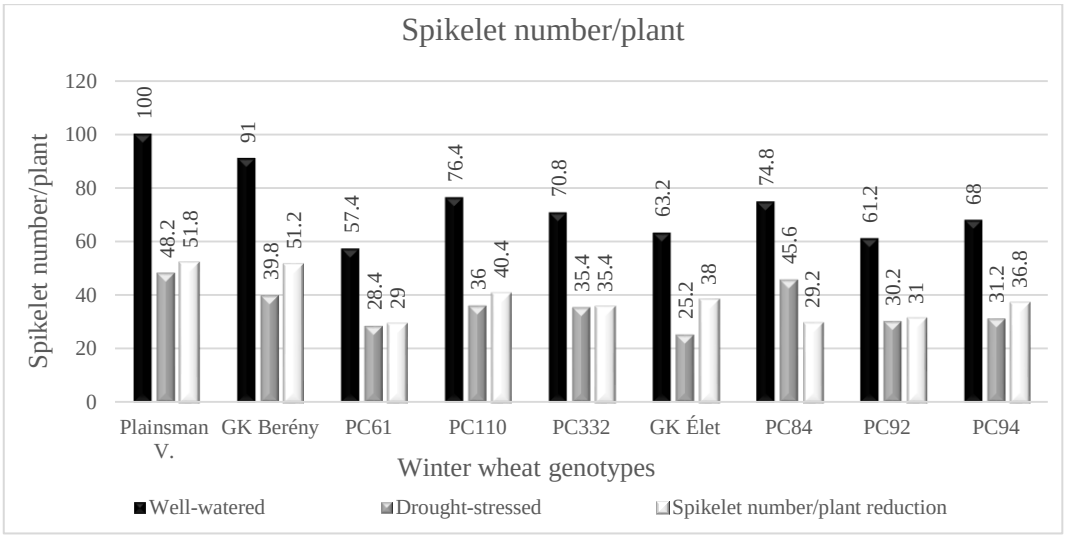

Figure 18. Spikelet number per plant of nine wheat genotypes under well-watered and drought stress conditions.

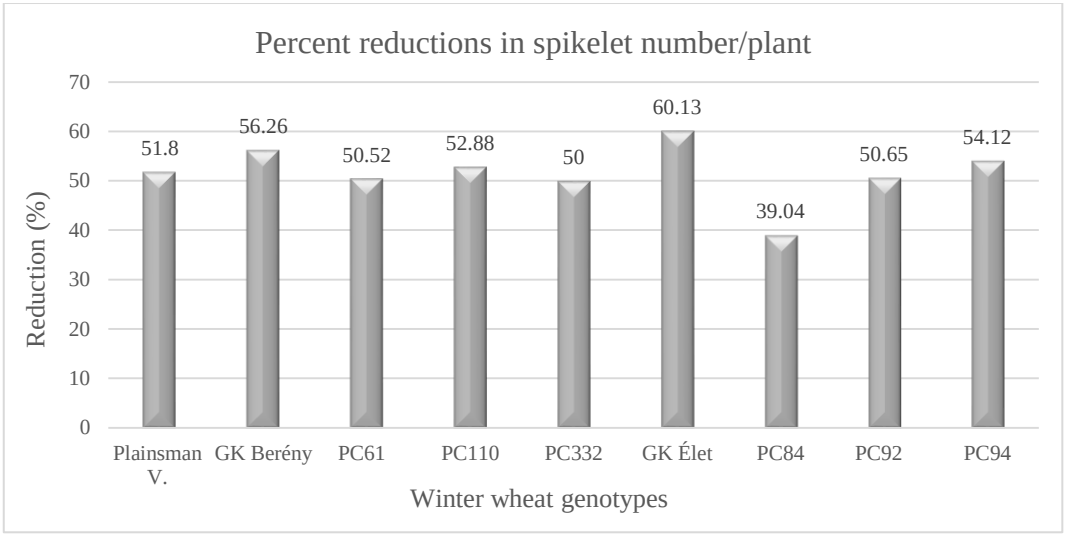

Figure 19. Percent reductions in spikelet number per plant affected by water deficit.

5.1.1.6. Fertile spikelet number per plant

Each genotype had a significant reduction in the fertile spikelet number per plant due to the drought effect compared with well-watered conditions. The values of fertile spikelet number/plant varied from 50.60 in ‘PC61’ to 87.60 in ‘Plainsman V.’ under well-watered conditions and from 13.40 in ‘PC94’ to 35.80 in ‘Plainsman V.’ under water deficit conditions.

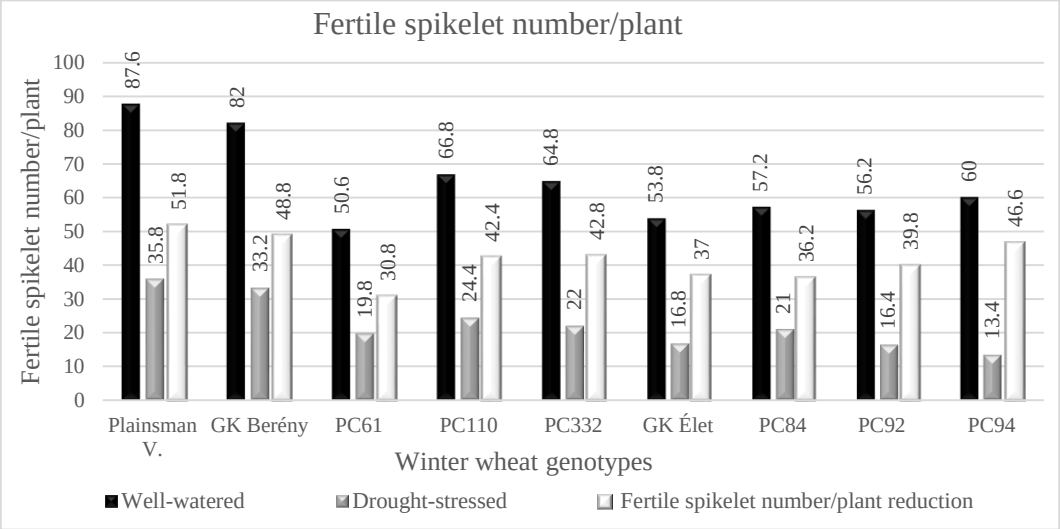

Figure 20. Fertile spikelet number per plant of nine wheat genotypes under well-watered and drought stress conditions.

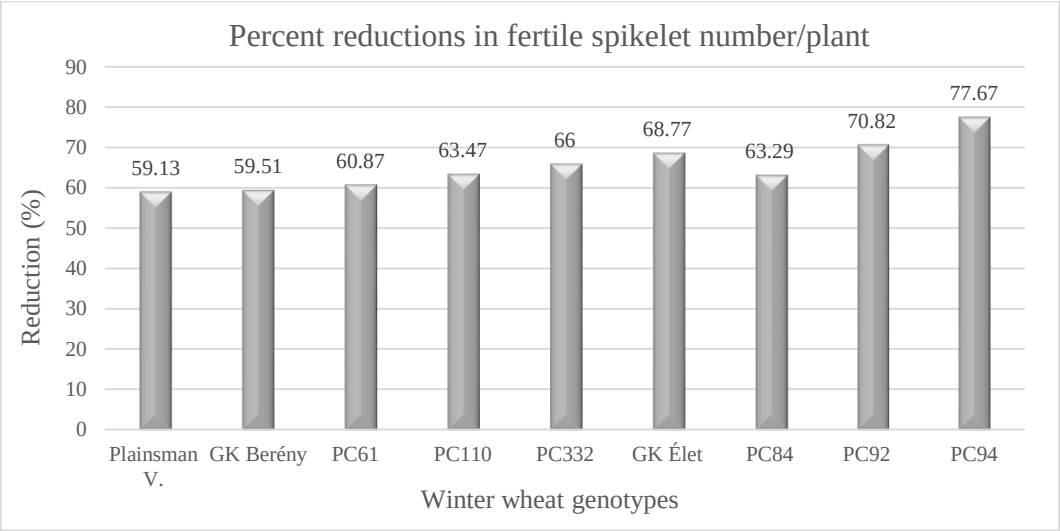

Figure 21. Percent reductions in fertile spikelet number per plant affected by water deficit.

The genotypes ‘PC61’, ‘PC84’ and ‘GK Élet’ showed the lowest reduction values for this trait (30.80, 36.20, and 37 fertile spikelet number/plant, respectively), while the genotypes ‘Plainsman V.’, ‘GK Berény’ and ‘PC94’ had the highest reduction values (51.80, 48.80 and 46.60 fertile spikelet number/plant, respectively) (Figure 20, Table 4).

The percent reductions of this trait ranged from 59.13% to 77.67% due to the drought stress compared to well-watered conditions; the lowest percent reductions were obtained in ‘Plainsman V.’, ‘GK Berény’ and ‘PC61’ (59.13, 59.51 and 60.87%, respectively), and the highest percent reductions were present in ‘PC94’, ‘PC92’ and ‘GK Élet’ (77.67, 70.82 and 68.77%, respectively) (Figure 21).

5.1.1.7. Grain number per plant

Water deficiency caused a significant drop in the grain number/plant of each investigated genotype; ‘PC84’ had the lowest variation of this trait, from 43.20 under drought stress to 128.40 under well-watered conditions, while ‘GK Berény’ showed the highest variance, from 68 under drought stress to 220.80 under well-watered conditions. The lowest decrease values of grain number/plant were obtained in ‘PC84’, ‘PC61’, and ‘GK Élet’ (58.20, 109.60 and 111.40, respectively), while the genotypes ‘GK Berény’, ‘PC332’ and ‘Plainsman V.’ had the highest decrease (152.80, 139 and 130.20, respectively) (Figure 22, Table 4).

The percent reductions of the grain number/plant of the genotypes varied from 64.84% to 79.01% under drought stress compared to well-watered conditions. The lowest percent reduction of the grain number/plant was present in the case of ‘Plainsman V.’, ‘PC84’ and ‘PC61’ (64.84, 66.36 and 67.57%, respectively), while the highest percent reductions (79.01, 78.20 and 77.02%) were obtained in the genotypes ‘GK Élet’, ‘PC94’ and ‘PC92’, respectively (Figure 23).

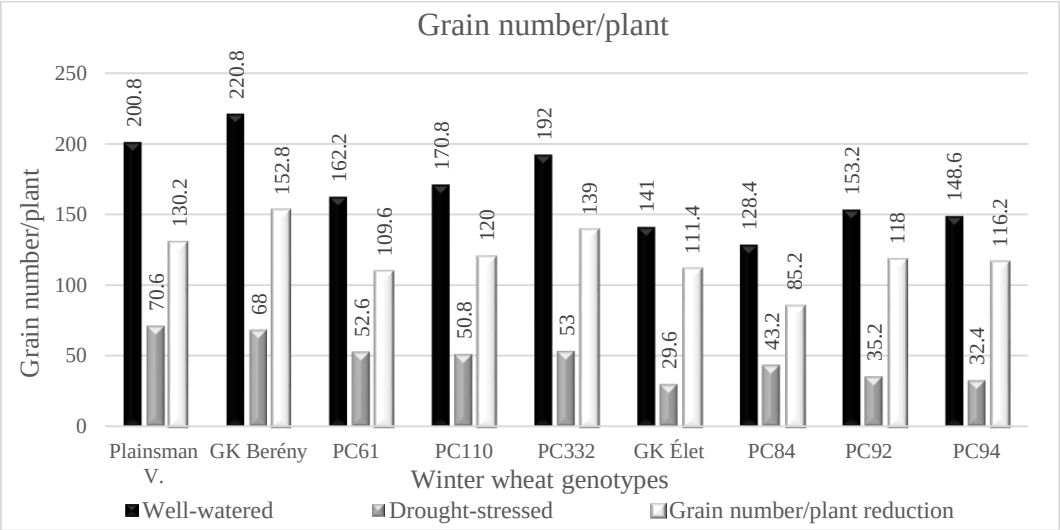

Figure 22. Grain number per plant of nine wheat genotypes under well-watered and drought stress conditions.

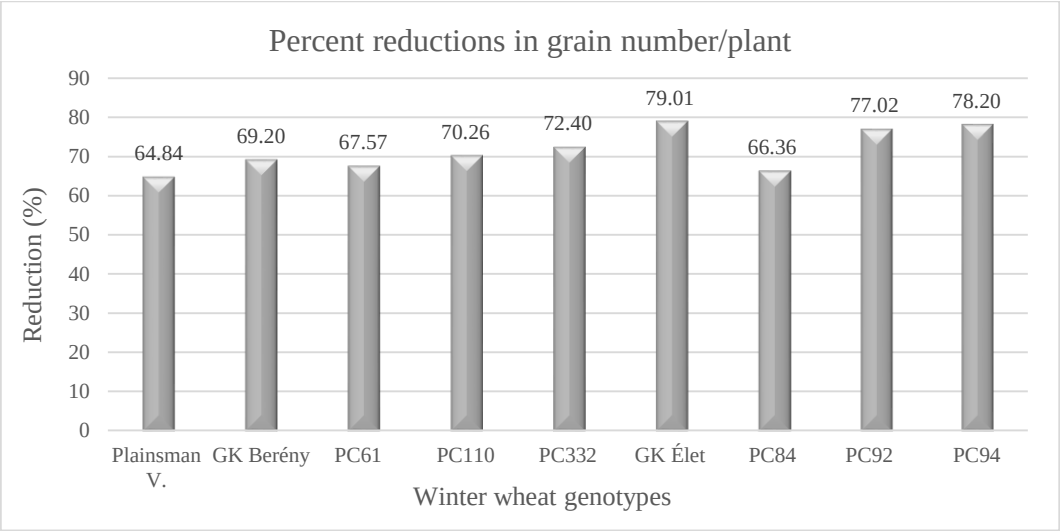

Figure 23. Percent reductions in grain number per plant affected by water deficit.

5.1.1.8. Grain yield per plant

The grain yield/plant of each investigated genotype decreased significantly under drought stress compared with the well-watered conditions. The values of grain yield/plant varied between 3.62 g in 'PC84' and 7.18 g in 'Plainsman V.' under well-watered conditions and between 0.93 g in 'PC94' and 2.18 g in 'Plainsman V.' under drought stress. The lowest decrease values of grain yield/plant were found in 'PC84', 'PC110' and 'PC94' (3.62, 3.90 and 4.16 g, respectively), while the genotypes 'GK Berény', 'Plainsman V.' and 'PC332' had the highest decrease values of grain yield/plant (5.08, 5.00 and 4.54 g, respectively) (Figure 24, Table 4).

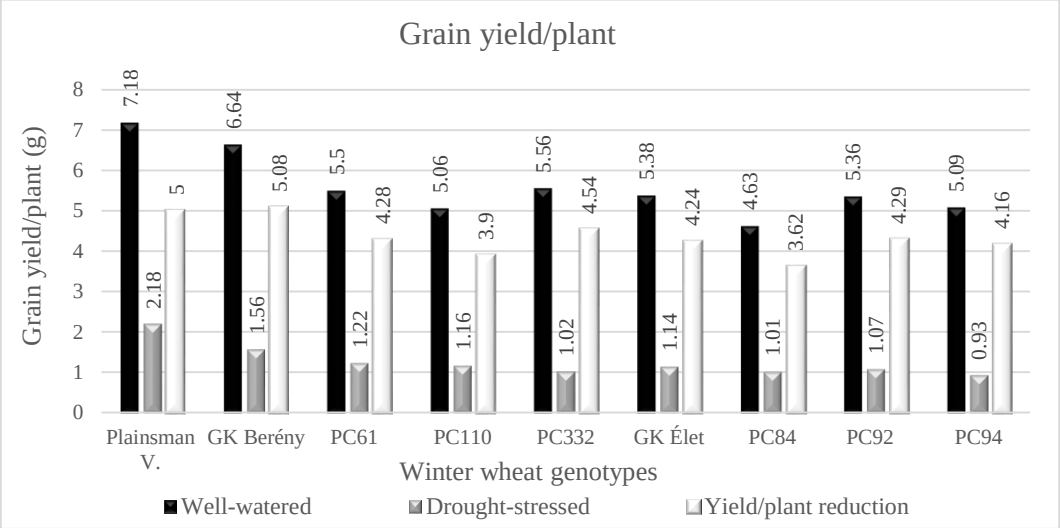

Figure 24. Grain yield per plant of nine wheat genotypes under well-watered and drought stress conditions.

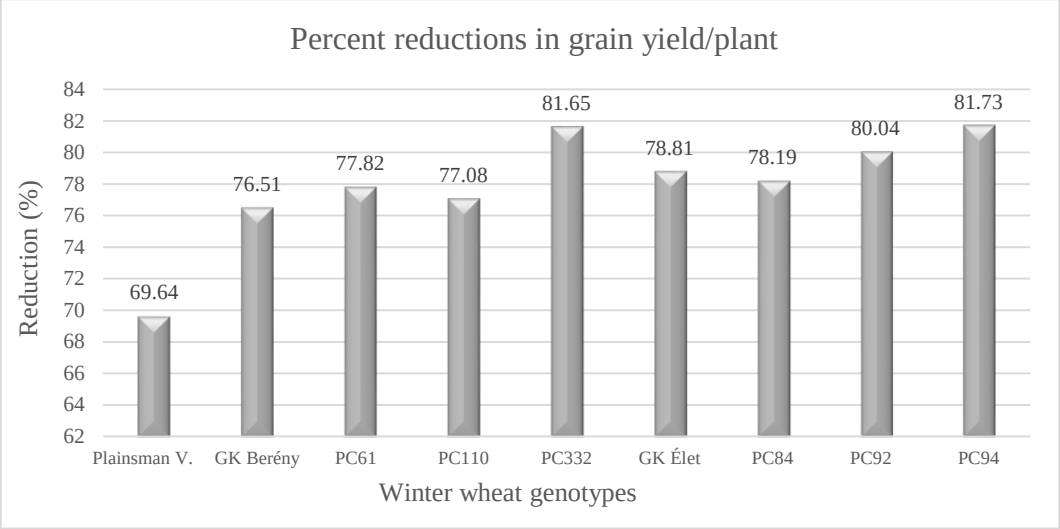

Figure 25. Percent reductions in grain yield per plant affected by water deficit.

In this investigation, the grain yield/plant performance of genotypes varied under the drought stress compared to the well-watered conditions, and the reduction percentage ranged from 69.64% to 81.73%. The genotypes 'Plainsman V.', 'GK Berény' and 'PC110' had the best performance of grain yield/plant according to their percent reduction index being the lowest among all values (69.64, 76.51 and 77.08%, respectively), while the highest grain yield/ plant loss percentages were present in 'PC94', 'PC332' and 'PC92' (81.73, 81.65 and 80.04%, respectively) (Figure 25). The calculated STI of the genotypes was between 0.298 and 0.179. The highest values of STI were observed in 'Plainsman V.', 'GK Berény', and 'PC61' (0.298, 0.261, and 0.214, respectively); these genotypes had higher STI than the drought-sensitive 'GK Élet' genotype (Table 5).

Table 5. Evaluation of the studied genotypes for drought tolerance depending on the tolerance indices calculated from the grain yield per plant values obtained in well-watered (WW) and drought stress (DS) treatments. (STI: stress tolerance index)

No	Genotype	Grain yield/plant		Reduction %	STI
		WW	DS		
1	Plainsman V.	7.18	2.18	69.64	0.298
2	GK Berény	6.64	1.56	76.51	0.261
3	PC61	5.50	1.22	77.82	0.214
4	PC110	5.06	1.16	77.08	0.198
5	PC332	5.56	1.02	81.65	0.210
6	GK Élet	5.38	1.14	78.81	0.208
7	PC84	4.63	1.01	78.19	0.179
8	PC92	5.36	1.07	80.04	0.205
9	PC94	5.09	0.93	81.73	0.191

5.1.1.9. Harvest index

All the studied genotypes responded to water deficiency with a harvest index reduction. The harvest index ranged between 45% in ‘Plainsman V.’ under drought stress and 49.71% under well-watered conditions – the lowest reduction – and ranged from 33.23% in ‘PC94’ under drought stress to 51.55% in well-watered conditions, representing the largest decrease.

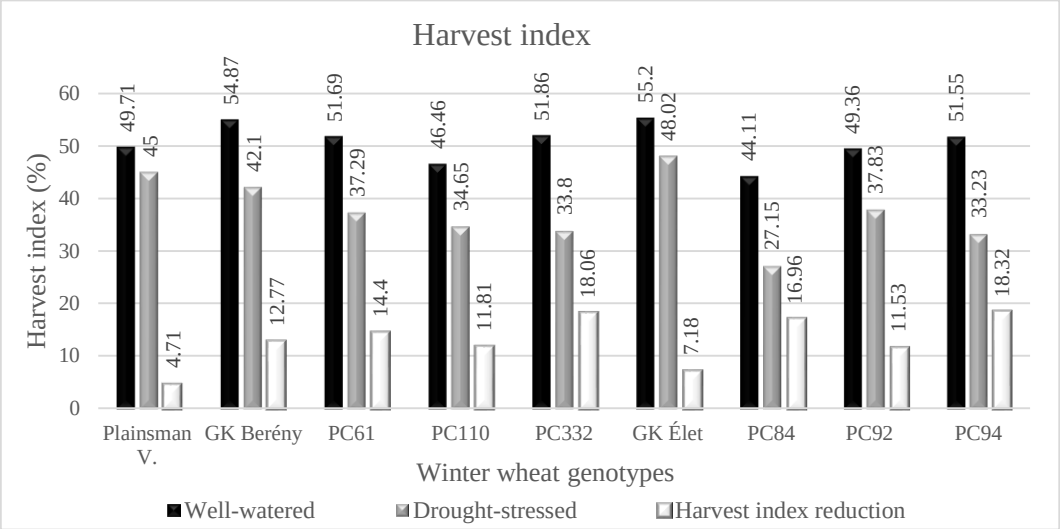

Figure 26. Harvest index of nine wheat genotypes under well-watered and drought stress conditions.

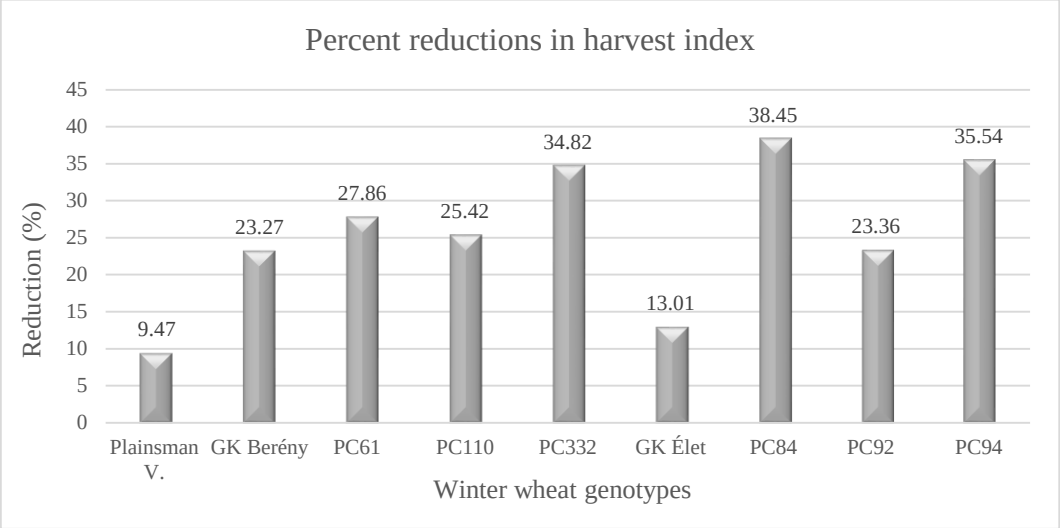

Figure 27. Percent reductions in harvest index affected by water deficit.

The genotypes ‘Plainsman V.’, ‘GK Élet’ and ‘PC92’ had the lowest decrease values of harvest index (4.71, 7.18 and 11.53%, respectively), while the largest decrease values were observed in ‘PC94’, ‘PC332’ and ‘PC84’ (18.32, 18.06 and 16.96%, respectively) (Figure 26, Table 4).

The percent reduction of the harvest index due to water deficiency was between 9.47% and 38.45%. The lowest percent reductions of this trait were in ‘Plainsman V.’, ‘GK Élet’ and ‘GK

Berény' (9.47, 13.01 and 23.27%, respectively), while the highest percent reductions were observed in the genotypes 'PC84', 'PC94' and 'PC332' (38.45, 35.54 and 34.82%, respectively) (Figure 27).

5.1.1.10. 1000-grain weight

Under well-watered conditions, the values of 1000-grain weight differed from 29.09 g in 'PC332' genotype to 38.51 in 'GK Élet' genotype, while the values were between 19.28 g in 'PC332' and 39.57 g in 'GK Élet' under drought stress conditions. All genotypes exposed 1000-grain weight reduction due to drought stress, except 'GK Élet'. The values of this reduction ranged between 3.70 g and 11.87 g. The genotypes 'PC92', 'Plainsman V.' and 'PC94' achieved the lowest reduction values (3.70, 4.96 and 5.29 g, respectively), while the genotypes 'PC84', 'PC332' and 'PC61' recorded the highest reduction values among all the tested genotypes (11.87, 9.81 and 9.54 g, respectively) (Figure 28, Table 4).

The percent reductions of 1000-grain weight varied between 10.48% in 'PC92' and 33.72% in 'PC332'. The 'GK Élet' genotype had no reduction under drought stress. The lowest percent reductions were recorded in 'PC92', 'Plainsman V.' and 'PC94' (10.48, 13.82, 15.47%, respectively), while the genotypes 'PC332', 'PC84' and 'PC61' had the highest percent reductions regarding this trait (33.72, 33.04 and 28.21%, respectively) (Figure 29).

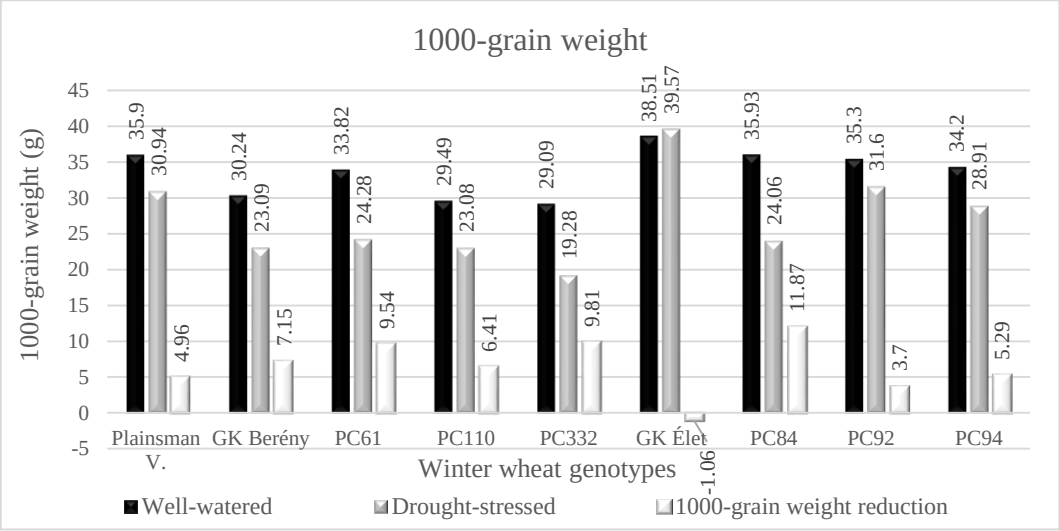

Figure 28. 1000-grain weight of nine wheat genotypes under well-watered and drought stress conditions.

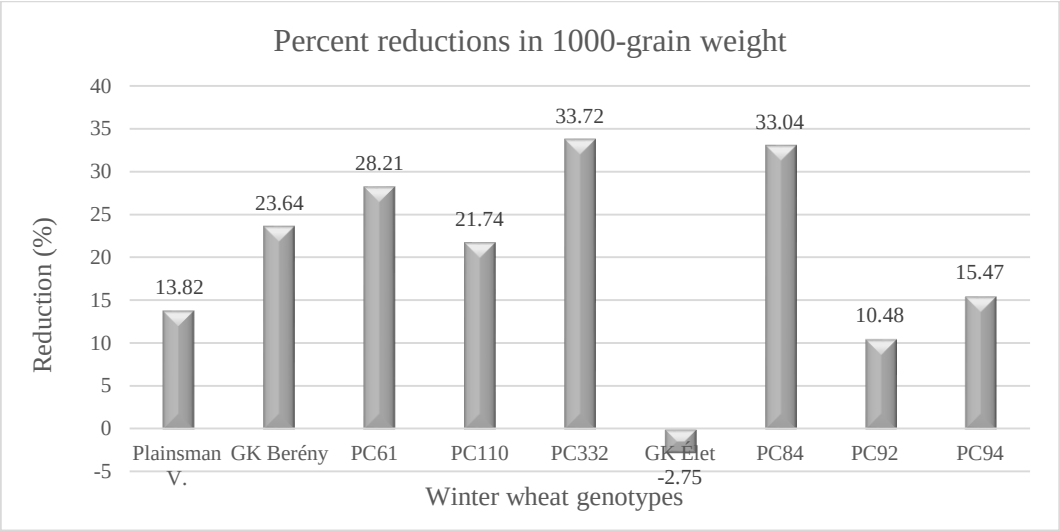

Figure 29. Percent reductions in1000-grain weight affected by water deficit.

5.1.1.11. Root length

The root length values varied from 18.20 cm in ‘GK Élet’ to 29.20 cm in ‘PC332’ under well-watered conditions, while the values ranged between 22.60 cm in ‘PC94’ and 27 cm in ‘PC61’ under drought stress conditions. Water deficit caused a non-significant root length decrease in ‘PC332’, ‘PC110’, ‘Plainsman V.’ and ‘PC84’ (3.60, 2.40, 0.60 and 0.40 cm, respectively), but the rest of the tested genotypes (GK Berény, PC94, PC92, PC61 and GK Élet) responded to water deficiency by increasing the root length. The increase was significant in ‘PC61’ and ‘GK Élet’ (7.40 and 8 cm, respectively) (Figure 30, Table 4).

Under drought stress, only four genotypes ‘PC84’, ‘Plainsman V.’, ‘PC110’ and ‘PC332’ showed root length reduction (1.63, 2.31, 8.28 and 12.33%, respectively) (Figure 31).

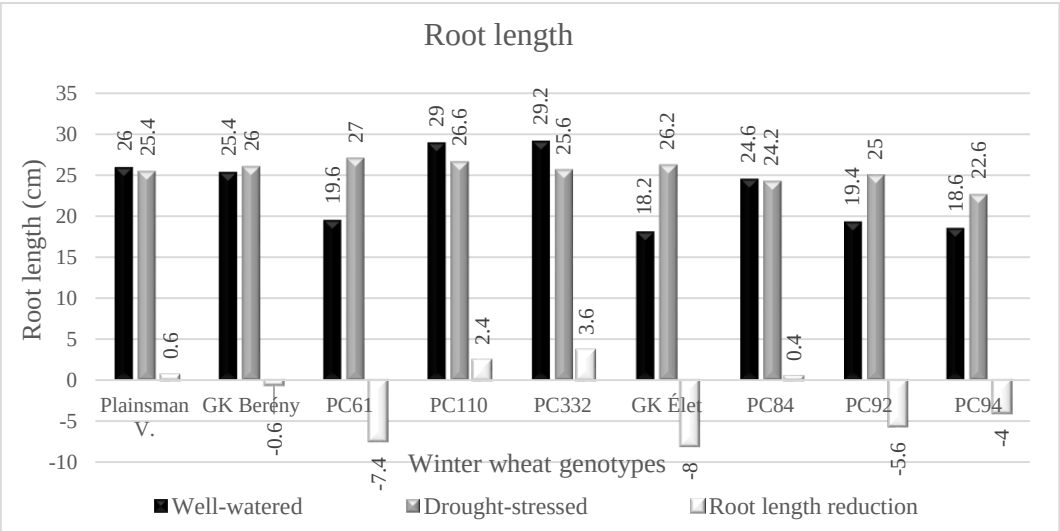

Figure 30. Root length of nine wheat genotypes under well-watered and drought stress conditions.

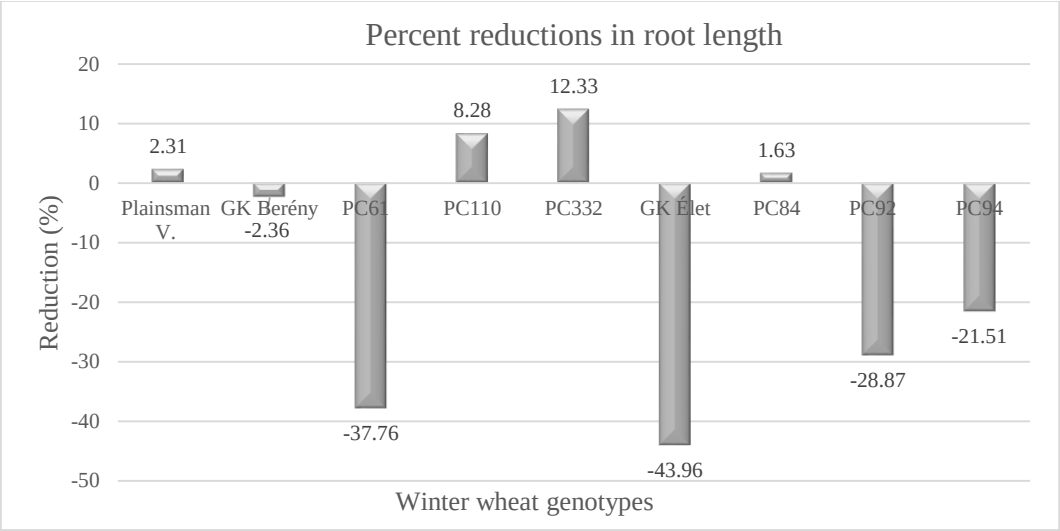

Figure 31. Percent reductions in root length affected by water deficit.

5.1.1.12. Root dry mass

Figure 34 and 35 illustrate the differences in root dry biomass of the studied genotypes under well-watered and drought stress conditions. A significant decrease was observed in the root dry mass trait of most genotypes under drought stress. While the genotypes 'PC94', 'PC61', 'PC110' had a non-significant reduction and the lowest reduction values (0.043, 0.075 and 0.079 g, respectively), whereas the highest reduction was found in 'Plainsman V.' and 'PC92' (0.241 and 0.195 g, respectively). Under well-watered conditions, plants attained root dry mass values from 0.171 g in 'PC94' to 0.481 g in 'Plainsman V.', while under drought stress conditions, plants had values between 0.072 g in 'GK Élet' and 0.240 g in 'Plainsman V.' (Figure 32, Table 4).

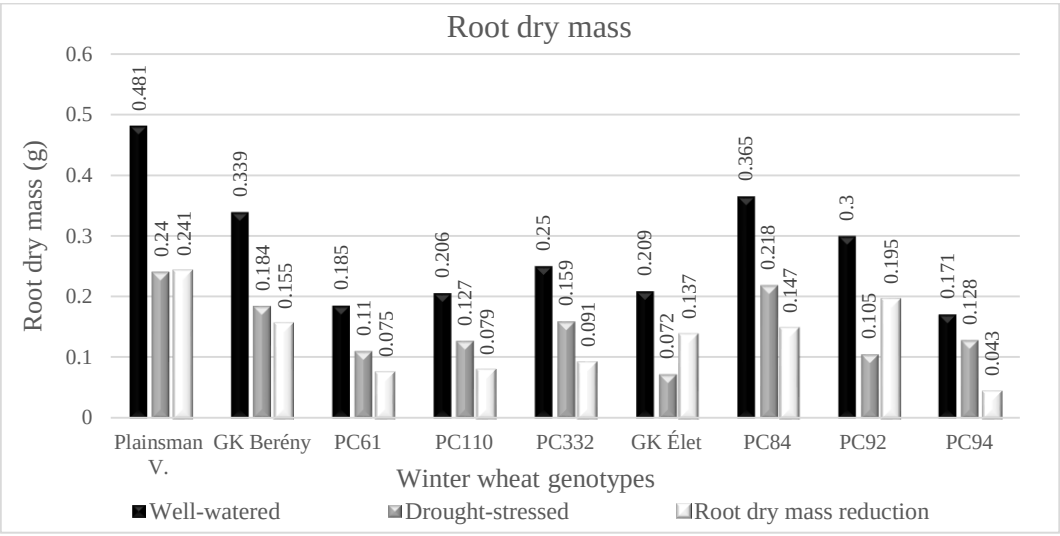

Figure 32. Root dry mass of nine wheat genotypes under well-watered and drought stress conditions.

The percentages of root dry mass loss caused by drought stress ranged between 25.15% and 65.55%. The smallest percent reductions of root dry mass under drought stress conditions were recorded in ‘PC94’, ‘PC332’ and ‘PC110’ (25.15, 36.40 and 38.35%, respectively), while the biggest percent reduction was in the genotypes ‘GK Élet’, ‘PC92’ and ‘Plainsman V.’ (65.55, 65 and 50.10%, respectively) (Figure 33).

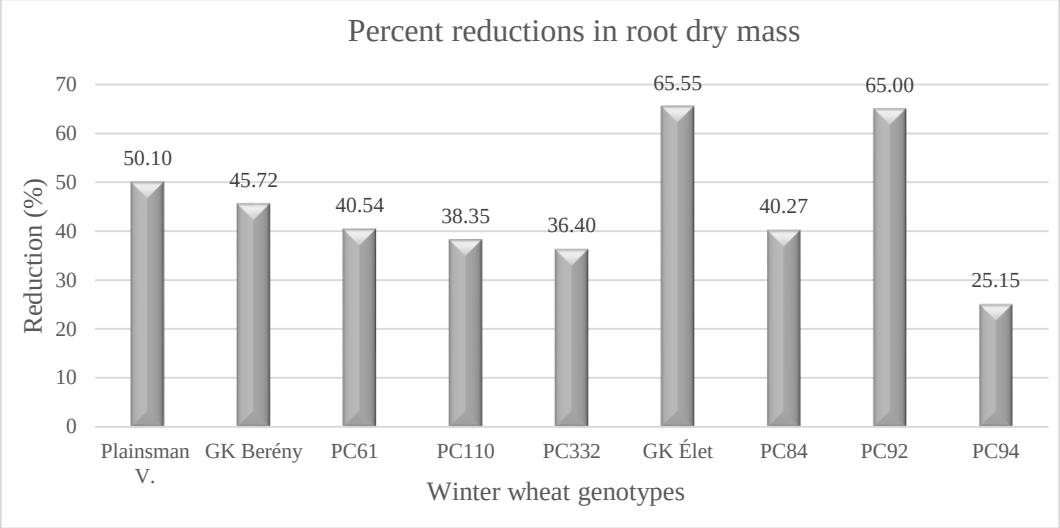

Figure 33. Percent reductions in root dry mass affected by water deficit.

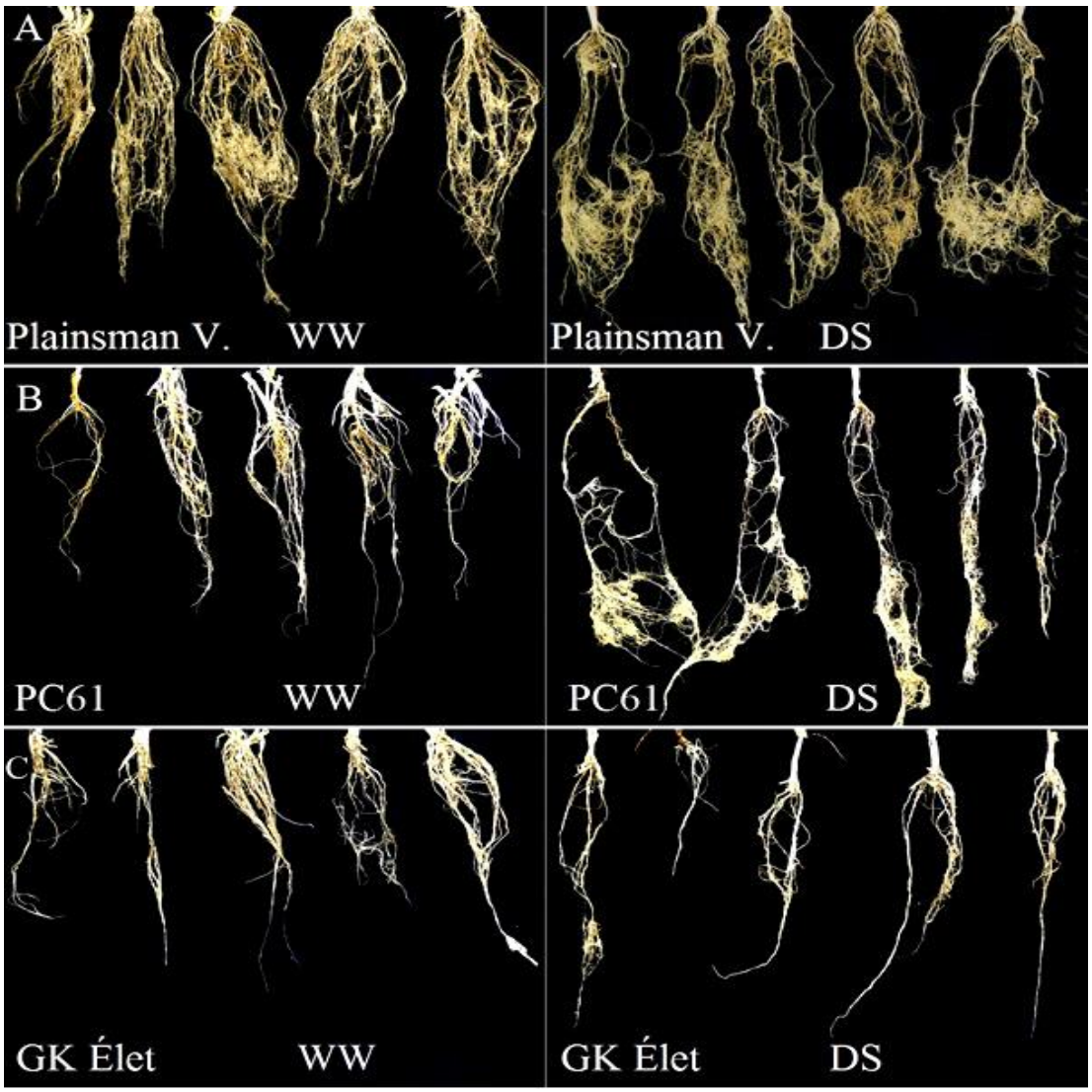

Figure 34. Comparison of the root dry biomass in the genotypes ‘Plainsman V.’ (A), ‘PC61’ (B) and ‘GK Élet’ (C) (five samples each) grown under well-watered (WW) and drought-stress (DS) conditions.

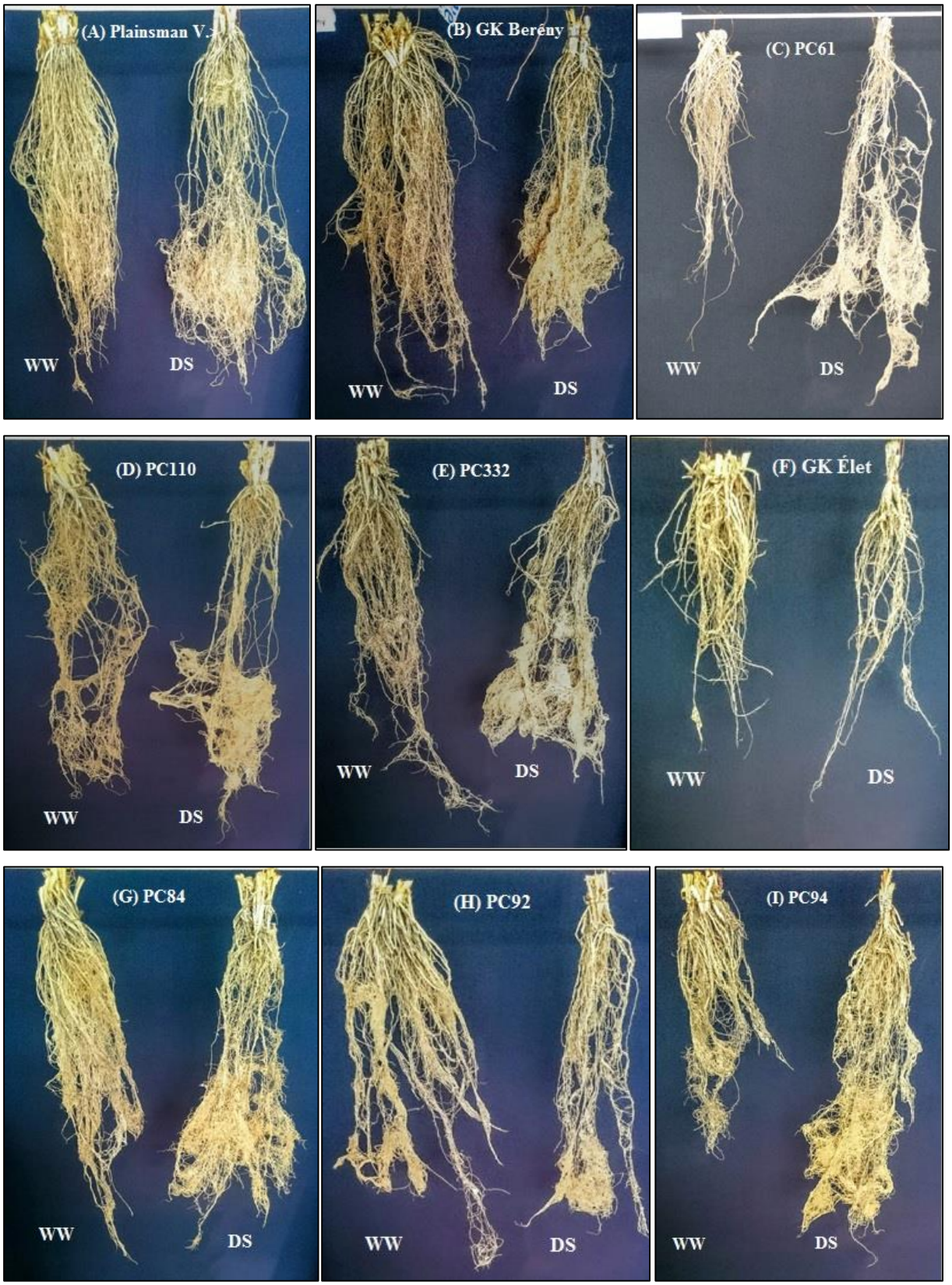

Figure 35. Comparison of the root dry biomass of all studied genotypes (A-I) grown under well-watered (WW) and drought-stress (DS) conditions. The presented samples contain 5 roots.

5.1.2. Correlation between the studied traits under well-watered and drought stress conditions

Table 6 demonstrates the correlation coefficient values for the studied traits. A positive significant correlation between heading time and above-ground biomass was obtained under well-watered and drought stress conditions, furthermore, heading time correlated significantly with grain yield/plant and plant height under drought stress. Plant height had a significant positive correlation with main spike length under drought stress conditions. Besides, above-ground biomass had a positive correlation with spikelet number/plant, fertile spikelet number/plant, grain yield/plant, root dry mass and grain number/plant under both conditions. Spikelet number/plant correlated positively and significantly with fertile spikelet number/plant, root dry mass and grain number/plant under both conditions, while there was a significant positive correlation between spikelet number/plant with grain yield/plant under drought stress conditions only. Fertile spikelet number/plant showed a significant positive correlation with grain number/plant, grain yield/plant and root dry mass under both treatments. Grain yield/plant showed a positive correlation with grain number/plant under both conditions, while root dry mass correlated positively with grain number/plant under drought stress. Grain yield/plant had a non-significant correlation with plant height, harvest index, 1000-grain weight, root length, and root dry mass, respectively, under both conditions.

On the other hand, a significant positive correlation was observed between grain yield/plant reduction and plant height, fertile spikelet number/plant, grain number/plant and harvest index reductions. A significant negative correlation was obtained between harvest index reduction and root dry mass reduction, while a significant positive correlation was observed between harvest index and plant height reduction. Furthermore, a positive significant correlation was observed between above-ground biomass reduction and both spikelet number/plant and grain number/plant reductions, and between grain number/plant and fertile spikelet number/plant reductions (Table 7).

Table 6. Correlation between all studied traits under well-watered (WW) and drought stress conditions (DS)

[ns: correlation is not significant, (*), (**), (***): correlation is significant at 0.05, 0.01, 0.001 probability levels, respectively. Traits abbreviations: HT, heading time; PH, plant height; AGB, above-ground biomass; MSL, main spike length; SPN/p, spikelet number/plant; FSN/p, fertile spikelet number/plant; GY/p, grain yield/plant; RDM, root dry mass; RL, root length; TGW, 1000-grain weight; GN/p, grain number/plant; HI, harvest index]

		HT	HT	PH	PH	AGB	AGB	MSL	MSL	SPN/p	SPN/p	FSN/p	FSN/p	GY/p	GY/p	RDM	RDM	RL	RL	TGW	TGW	GN/p	GN/p
		WW	DS	WW	DS	WW	DS	WW	DS	WW	DS	WW	DS	WW	DS	WW	DS	WW	DS	WW	DS	WW	DS
HT	WW																						
HT	DS	0.927***																					
PH	WW	ns	ns																				
PH	DS	0.788*	.872**	0.680*																			
AGB	WW	0.747**	.860**	ns	0.674*																		
AGB	DS	0.838**	.830**	ns	ns	0.91***																	
MSL	WW	ns	ns	ns	0.686*	ns	ns																
MSL	DS	ns	ns	0.681*	0.855**	ns	ns	0.918**															
SPN/p	WW	ns	ns	ns	ns	0.852**	0.839**	ns	ns														
SPN/p	DS	0.740*	ns	ns	ns	0.726*	0.888**	ns	ns	0.843**													
FSN/p	WW	ns	ns	ns	ns	0.869**	0.717*	ns	ns	0.964*	0.717*												
FSN/p	DS	ns	ns	ns	ns	0.893**	0.858**	ns	ns	0.910***	0.743*	0.909***											
GY/p	WW	ns	n	ns	ns	0.88**	0.667*	ns	ns	0.742*	ns	0.836**	0.826**										
GY/p	DS	ns	).795*	ns	ns	0.953***	0.836**	ns	ns	0.813**	ns	0.826**	0.874**	0.918***									
RDM	WW	ns	).697*	ns	ns	0.844**	0.836**	ns	ns	0.780*	0.847**	0.699*	0.740*	ns	0.749*								
RDM	DS	0.703*	ns	ns	ns	0.73*	0.887**	ns	ns	0.819**	0.978***	0.706*	0.726*	ns	ns	0.839**							
RL	WW	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns						
RL	DS	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns					
TGW	WW	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	-0.712*	ns				
TGW	DS	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	-0.686*	ns	0.840**			
GN/p	WW	ns	ns	ns	ns	0.70*	ns	ns	ns	0.689*	ns	0.830**	0.812**	0.832**	ns	ns	ns	ns	ns	ns	ns		
GN/p	DS	ns	ns	ns	ns	0.84**	0.838**	ns	ns	0.785*	0.673*	0.818**	0.940***	0.871*	0.784*	ns	0.695*	ns	ns	ns	ns	0.861**	
HI	WW	ns	ns	ns	ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
HI	DS	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.676*	ns	ns	ns	ns	ns	ns	ns	ns	ns

Table 7. Correlations between plant height reduction (PH.R), above-ground biomass reduction (AGB.R), main spike length reduction (MSL.R), spikelet number/plant reduction (SPN/p.R), fertile spikelet number/plant reduction (FSN/p.R), grain number/plant reduction (GN/p.R), grain yield/plant reduction (GY/p.R), harvest index reduction (HI.R), root dry mass reduction (RDM.R) [ns: correlation is not significant, (*), (**), (***): correlation is significant at the 0.05, 0.01, 0.001 probability levels, respectively]

	PH.R	AGB.R	MSL.R	SPN/p.R	FSN/p.R	GN/p.R	GY/p.R	HI.R
PH.R								
AGB.R	ns							
MSL.R	ns	ns						
SPN/p.R	ns	0.688*	ns					
FSN/p.R	ns	ns	ns	ns				
GN/p.R	ns	0.913***	ns	ns	0.871**			
GY/p.R	0.816**	ns	ns	ns	0.712*	0.687*		
HI.R	0.705**	ns	ns	ns	ns	ns	0.685*	
RDM.R	ns	ns	ns	ns	ns	ns	ns	-0.70*

5.1.3. Relationships between some studied traits under well-watered and drought stress conditions

Simple linear regression analysis revealed the relationships between some of the studied traits (Figure 36). Under well-watered conditions, strong significant relationships were observed between the grain yield/plant with both above-ground biomass (Figure 36A) and grain number/plant (Figure 36B). Furthermore, above-ground biomass showed a strong significant relationship with root dry mass (Figure 36D); while a non-significant relationship was obtained between root dry mass and grain yield/plant (Figure 36C). On the other hand, under drought stress, moderate relationships were found between grain yield/plant and both heading time and grain number/plant (Figures 36E and G). There was a strong and significant relationship between grain yield/plant and above-ground biomass (Figure 36F), while the relationship between grain yield/plant and root dry mass was non-significant (Figure 36H). Root dry mass and above-ground biomass showed a strong positive significant relationship under drought stress (Figure 36I).

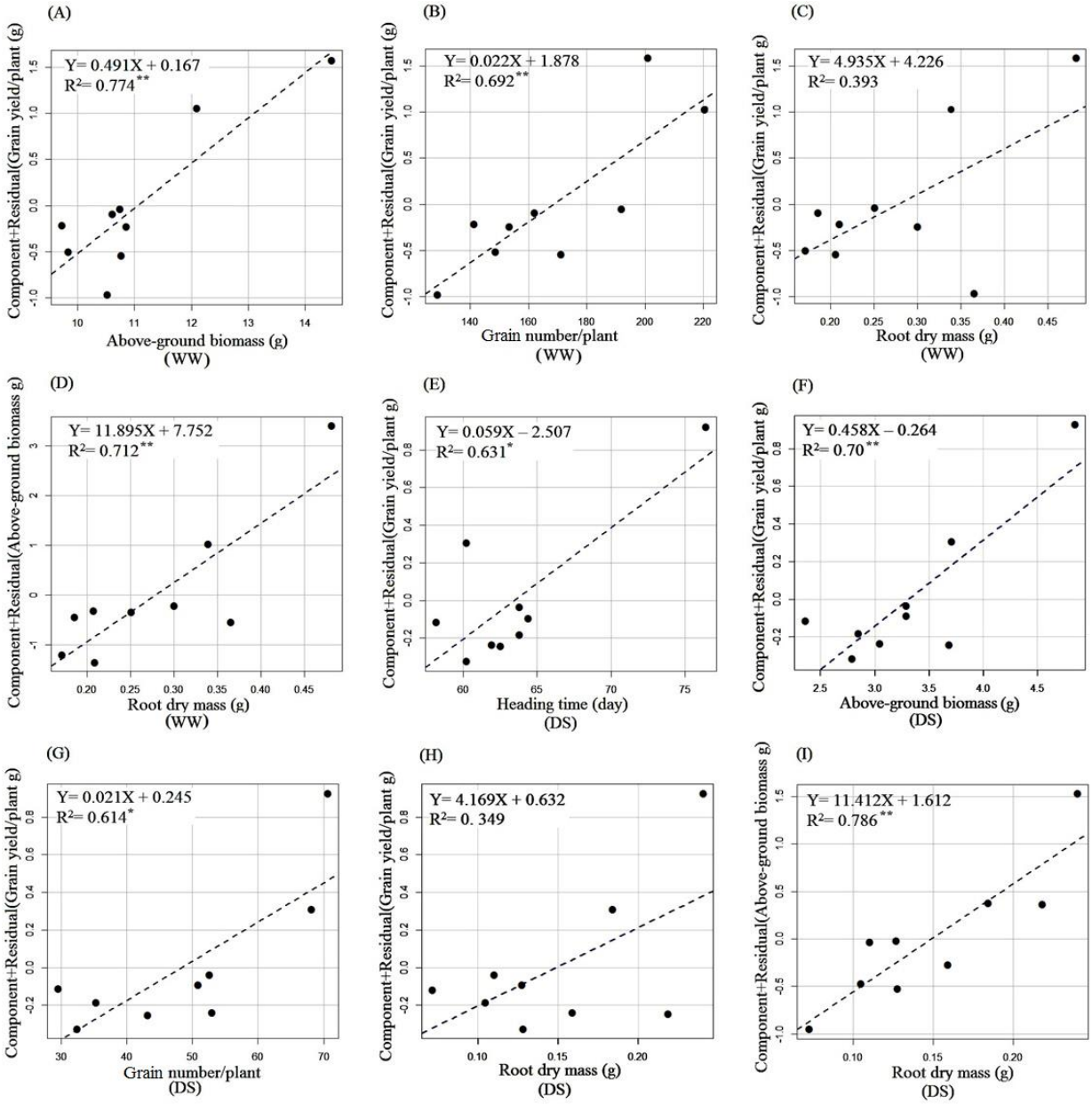

Figure 36. Simple relationships between some traits in the case of well-watered (WW) and drought-stress (DS) treatments: (A), relationship between AGB and GY/p under WW treatment; (B), relationship between GN/p and GY/p under WW treatment; (C), relationship between RDM and GY/p under WW treatment; (D), relationship between RDM and AGB under WW treatment; (E), relationship between HT and GY/p under DS treatment; (F) relationship between AGB and GY/p under DS treatment; (G) relationship between GN/p and GY/p under DS treatment; (H) relationship between RDM and GY/p under DS treatment; (I) relationship between RDM and AGB under DS treatment. Traits abbreviations: see Table 6.

5.2. Generation of winter wheat doubled haploid lines via *in vitro* anther culture

The statistical analysis showed that the effect of the genotype was significant for all the investigated androgenetic parameters – the number of embryo-like structures, regenerated-, green-, albino-, and transplanted plantlets – at the $P < 0.001$ probability level (Table 8).

Table 8. Statistical analysis of the androgenetic parameters for thirteen wheat F_4 combinations by the one-way ANOVA

(*** The values significantly differ at the P < 0.001 probability level, SS: sum of squares, MS: mean square, DF: degree of freedom, Pr: probability)

Androgenetic parameters /100 anthers	Source of variance	DF	SS	MS	F value	Pr (>F)
Number of embryo-like structures	Combination	12	64186	5349***	19.68	<0.0000
	Error	111	30173	272		
Number of regenerated plantlets	Combination	12	15958	1329.8***	22.66	<0.0000
	Error	111	6513	58.7		
Number of green plantlets	Combination	12	7848	654 ***	21.03	<0.0000
	Error	111	3452	31.1		
Number of albino plantlets	Combination	12	5281	440.1 ***	26.80	<0.0000
	Error	111	1823	16.4		
Number of transplanted plantlets	Combination	12	2172	180.96 ***	13.07	<0.0000
	Error	111	1537	13.85		

5.2.1. Evaluation of androgenetic traits of winter wheat F_4 combinations in anther culture

5.2.1.1. The number of embryo-like structures per 100 anthers

Table 9 summarises the significant differences among the genotypes for all the studied traits. The number of the embryo-like structures per 100 anthers varied between 6.0 and 74.5, depending on the combination (genotype). The highest values were recorded in the combinations, namely, ‘Béres/Pamier’, ‘Kalász/Tacitus’, and ‘Midas/Csillag//Tacitus/5003’ (74.4, 71.0, 65.3, respectively). The lowest values were in the combinations, ‘Kolo/Premio’, ‘Kapos/Ködmön’, and ‘DH54/12.189’ (6.0, 6.4, 13.1, respectively). The overall mean of the 13 F_4 combinations was 35.84 embryo-like structures/100 anthers (Table 9).

5.2.1.2. The number of regenerated plantlets per 100 anthers

The number of the regenerated plantlets per 100 anthers ranged between 0.6 in the combination ‘Kolo/Premio’ and 36.3 in ‘Kalász/Tacitus’. The combinations ‘Kalász/Tacitus’, ‘Béres/Pamier’, and ‘Premio/5009’ had the highest values (36.3, 30.9, 26.3, respectively), while the combinations ‘Kolo/Premio’. ‘Kapos/Ködmön’ and ‘DH54/12.189’ had the lowest values (0.6, 2.5, 3.8, respectively). The overall mean of the 13 combinations was 13.9 regenerated plantlets/100 anthers (Table 9).

Green plantlets regenerated from the embryo-like structures of all crossing combinations. The number of green plantlets per 100 anthers varied between 0.4 and 24.7. The combinations ‘Premio/5009’, ‘Béres/Midas’, and ‘Béres/Pamier’ showed the highest values of green plantlets per 100 anthers (24.7, 22.1, and 15.9, respectively) while the combinations ‘Kolo/Premio’, ‘Kapos/Ködmön’ and ‘DH54/12.89’ had the lowest values (0.4, 0.9 and 1.5, respectively). The overall mean of the combinations was 8.3 green plantlets/100 anthers (Table 9).

Albino plantlets were found in each combination. The values per 100 anthers were between 0.2 and 22.8. The highest values were obtained in the combinations ‘Kalász/Tacitus’, ‘Béres/Pamier’ and ‘Körös/Premio’ (22.8, 14.9, 10.2 albino plantlets/100 anthers, respectively), while the lowest values were in the combinations ‘Kolo/Premio’, ‘DH54/12.189’ and ‘DL45/DH150’ (0.2, 1.5, 1.5 albinos/100 anthers, respectively). In this experiment, the overall mean value was 5.6 albino plantlets/100 anthers (Table 9).

5.2.1.3. The number of transplanted plantlets per 100 anthers

The values of the transplanted plantlets per 100 anthers ranged between 0.3 and 12.6 transplanted plantlets/100 anthers depending on the combination. The combinations: ‘Béres/Midas’, ‘Béres/Pamier’ and ‘Premio/5009’ achieved the highest values (12.6, 11.4, 9.7, respectively), whereas the combinations ‘Kolo/Premio’, ‘Kapos/Ködmön’ and ‘Körös/Premio’ had the lowest values (0.3, 0.7, 1.0, respectively), while the overall mean of this parameter was 5.2 transplanted plantlets/100 anthers (Table 9).

Table 9. Androgenetic responses of thirteen wheat F_4 combinations in anther culture. The values followed by the same letters within a column are not significantly different at the P = 0.05 probability level as determined by the pairwise comparison of means test (Tukey Contrasts)
[(SD) Standard deviation of the mean, (SE) Standard error of the mean, (CV) Coefficient of variation, (LSD) Least significant difference]

Code of combinations	Embryo-like structures /100 anthers	Regenerated plantlets /100 anthers	Green plantlets /100 anthers	Albino plantlets /100 anthers	Transplanted plantlets /100 anthers
2522	20.3^{ce}	9.6^{d}	5.2^{def}	4.4^{de}	4.3^{cdf}
2533	44.1^{bc}	26.3^{ab}	24.7^{a}	1.6^{e}	9.7^{abc}
2570	34.4^{cd}	12.5^{cd}	6.7^{cf}	5.8^{de}	5.2^{bef}
2572	25.5^{ce}	10.5^{d}	9.0^{ce}	1.5^{e}	6.4^{bde}
2581	39.2^{c}	23.8^{bc}	22.1^{ab}	1.7^{e}	12.6^{a}
2591	74.5^{a}	30.9^{ab}	15.9^{bc}	14.9^{c}	11.4^{ab}
2610	71.0^{a}	36.3^{a}	13.5^{cd}	22.8^{b}	8.8^{abd}
2635	6.0^{e}	0.6^{d}	0.4^{f}	0.2^{e}	0.3^{f}
2680	39.7^{c}	12.0^{d}	1.8^{ef}	10.2^{cd}	1.0^{ef}
2712	65.3^{ab}	6.6^{d}	3.7^{ef}	2.9^{e}	3.4^{df}
2739	13.1^{de}	3.8^{d}	2.4^{ef}	1.5^{e}	1.5^{ef}
2740	24.5^{ce}	4.3^{d}	1.5^{ef}	2.8^{e}	1.3^{ef}
2744	6.4^{e}	2.5^{d}	0.9^{ef}	1.6^{e}	0.7^{ef}
Mean	35.8	13.9	8.3	5.6	5.2
SD	27.7	13.5	9.6	7.6	5.5
SE	2.5	1.2	0.9	0.7	0.5
CV	0.7728	0.9724	1.1534	1.3592	1.065
$LSD_{0.05}$	15.5	7.2	5.2	3.8	3.5

5.2.2. The efficiency of green plantlet production per 100 embryo-like structures and 100 regenerated plantlets in anther culture

The results of the statistical analysis revealed that the genotype (combination) had a significant effect on the green plantlets per 100 embryo-like structures, albino plantlets per 100 embryo-like structures, green plantlets per 100 regenerated plantlets, and albino plantlets per 100 regenerated plantlets at the $P < 0.001$ probability level (Table 10).

Table 10. Statistical analysis of the androgenetic parameters regenerated plantlets, green plantlets, and albino plantlets per 100 embryo-like structures and 100 regeneration plantlets for thirteen wheat F_4 combinations by the one-way ANOVA

(*** The values significant at the $P < 0.001$ probability level, DF: degree of freedom, SS: sum of squares, MS: mean square, Pr: probability)

Androgenetic parameters /100 embryo-like structures	Source of variance	DF	SS	MS	F value	Pr (>F)
Number of regenerated Plantlets	Combination	12	34521	2876.7***	14.35	<0.0000
	Error	111	22248	200.4		
Number of green plantlets	Combination	12	34211	2850.9***	30.47	<0.0000
	Error	111	10385	93.6		
Number of albino plantlets	Combination	12	13653	1137.7***	11.74	<0.0000
	Error	111	10758	96.9		
Androgenetic parameters /100 regenerated plantlets	Source of variance	DF	SS	MS	F value	Pr (>F)
Number of green plantlets	Combination	12	64114	5343***	16.18	<0.0000
	Error	108	35663	330		
Number of albino plantlets	Combination	12	64114	5343***	16.18	<0.0000
	Error	108	35663	330		

The number of green plantlets/ 100 embryo-like structures ranged from 4.9 in the combination 'DH54/12.89' to 56.0 in the combination 'Béres/Midas', while the overall mean was 22.7 (Table 11). An average of 15.3 albino plantlets/100 embryo-like structures varied from 3.6 in the 'Kolo/Premio' combination to 34.7 in 'Kalász/Tacitus' combination (Table 11).

The number of green plantlets/100 regenerated plantlets ranged between 16.2 and 93.3 depending on the combination, with a mean of 56.1. The combinations 'Béres/Midas', 'Premio/5009' and 'DL45/DH150' achieved the highest values of green plantlets/100 embryo-like structures (56.0, 55.9 and 35.6, respectively), and had the highest numbers of green plantlets/100 regenerated plantlets, but in a different order: 'Premio/5009', 'Béres/Midas' and 'DL45/DH150'– 93.3, 92.4 and 84.3 green plantlets/100 regenerated plantlets, respectively (Table 11). The values of albino plantlets/100 regenerated plantlets were between 6.8 in the combination 'Premio/5009' and 83.8 in the combination 'Körös/Premio', with an overall mean of 43.9 (Table 11).

Table 11. Statistical analysis of the parameters regenerated plantlets, green plantlets and albino plantlets per 100 embryo-like structures and 100 regenerated plantlets for thirteen wheat F_4 combinations in anther culture.

[The values followed by the same letters within a column are not significantly different at the P = 0.05 probability levels as determined by the pairwise comparison of means test (Tukey Contrasts) [(SD) Standard deviation of the mean, (SE) Standard error of the mean, (CV) Coefficient of variation, (LSD) Least significant difference]

No	Combination code	Number of regenerated plantlets /100 embryo-like structures	Number of green plantlets /100 embryo-like structures	Number of Albino plantlets /100 embryo-like structures	Number of green plantlets/100 regenerated plantlets	Number of albino plantlets/100 regenerated plantlets
1	2522	53.2^{a}	26.1^{bc}	27.1^{ab}	49.3^{ce}	50.7^{cd}
2	2533	60.1^{a}	55.9^{a}	4.1^{f}	93.3^{a}	6.8^{f}
3	2570	38.7^{abc}	21.4^{bdf}	17.3^{bdf}	59.2^{bce}	40.8^{cde}
4	2572	41.4^{ab}	35.6^{b}	5.7^{ef}	84.3^{ab}	15.7^{ef}
5	2581	60.6^{a}	56.0^{a}	4.6^{f}	92.4^{a}	7.7^{f}
6	2591	41.0^{ab}	21.2^{bd}	19.7^{bcde}	50.2^{ce}	49.8^{cd}
7	2610	53.9^{a}	19.2^{cde}	34.7^{a}	35.5^{def}	64.5^{abc}
8	2635	11.0^{d}	7.4^{de}	3.6^{f}	69.7^{ac}	30.3^{df}
9	2680	31.0^{bd}	5.2^{e}	25.8^{acd}	16.2^{f}	83.8^{a}
10	2712	11.4^{d}	6.1^{ef}	5.3^{ef}	54.0^{ce}	46.0^{cd}
11	2739	29.0^{bd}	17.1^{cde}	11.9^{cf}	58.1^{bcd}	42.0^{bde}
12	2740	15.9^{cd}	4.9^{ef}	11.0^{df}	28.7^{ef}	71.3^{ac}
13	2744	44.7^{ab}	17.8^{cde}	26.8^{ad}	41.3^{cf}	58.8^{ad}
Mean		38.0	22.7	15.3	56.1	43.9
SD		21.5	19.0	14.1	28.8	28.8
SE		1.9	1.7	1.3	2.6	2.6
CV		0.5655	0.8404	0.9195	0.5142	0.6564
$LSD_{0.05}$		13.3	9.1	9.3	17.1	17.1

5.2.3. Production of doubled haploid lines

A total of 1545 acclimatized plantlets were obtained in this experiment (Table 12). The highest values were found in the combinations ‘Béres/Midas’, ‘Béres/Pamier’, ‘Kalász/Tacitus’, and ‘Premio/5009’ (301, 270, 239, and 194, respectively). In total, 923 spontaneous doubled haploids were recovered in the nursery with an overall mean of 59.7/100 acclimatised plantlets. The rate of doubled haploid/100 acclimatised plantlets ranged between 25% and 87.8% across the combinations. The highest number of doubled haploid plants were found in the combinations ‘Béres/Midas’, ‘Kalász/Tacitus’, ‘Béres/Pamier’, and ‘Premio/5009’÷ (191, 183, 127, and 120, respectively).

Table 12. Production of spontaneous doubled haploid plants from thirteen wheat F_4 breeding combinations via anther culture

Combinations	Total number of green plantlets	Total number and percentage of transplanted plantlets		Total number and percentage of acclimatised plantlets		Number and percentage of doubled haploid plants					
						Fertile		Partially fertile		Total number and percentage	
		No	%	No	%	No	%	No	%	No	%
2522	156	130	83.3	83	63.9	15	18.1	28	33.7	43	51.8
2533	668	261	39.1	194	74.3	15	7.7	105	54.1	120	61.9
2570	144	110	76.4	88	80.0	15	17.1	22	25.0	37	42.1
2572	270	191	70.7	146	76.4	40	27.4	49	33.6	89	61.0
2581	664	378	56.9	301	79.6	84	27.9	107	35.6	191	63.5
2591	478	343	71.8	270	78.7	87	32.2	40	14.8	127	47.0
2610	404	265	65.6	239	90.2	51	21.3	132	55.2	183	76.6
2635	11	10	90.9	8	80.0	2	25.0	0.0	0.0	2	25.0
2680	54	30	55.6	29	96.7	5	17.2	5	17.2	10	34.5
2712	111	101	91.0	99	98.0	18	18.2	28	28.3	46	46.5
2739	71	53	74.7	49	92.5	12	24.5	31	63.3	43	87.8
2740	36	32	88.9	31	96.9	8	25.8	19	61.3	27	87.1
2744	27	22	81.5	8	36.4	5	62.5	0.0	0.0	5	62.5
Total number	3094	1926	-	1545	-	357	-	566	-	923	-
Overall mean	-	-	62.0	-	80.1	-	23.1	-	36.6	-	59.7

6. DISCUSSION

6.1. Characterization of winter wheat genotypes for drought tolerance

The global agricultural sector has been facing main difficulties and challenges arising from climate change realities, but at the same time, the need to produce 70% more food for the planet's rapidly growing population is highly urgent. The mentioned and some other factors impede crop productivity, thus crippling the efforts to meet the food demand. Drought is one of the environmental factors that reduce cereal crop production worldwide (RIVERO et al. 2007; PARIHAR et al. 2015; RAMYA et al. 2016). Breeders try to overcome this obstacle through developing, phenotyping and selecting new drought-tolerant genotypes (GRZESIAK et al. 2019).

Shoot dry weight and grain yield parameters measured after harvest are relevant traits in the characterization of wheat genotypes for drought tolerance (MAJER et al. 2008). The relative grain yield performance of genotypes under well-watered and drought stress conditions is considered as an essential onset point to identify the traits associated with drought resistance and the selection of drought-tolerant genotypes (SIO-SE MARDEH et al. 2006). Subsequently, the groups of target traits associated with grain yield under drought stress should be selected for drought tolerance trials (MWADZINGENI et al. 2016b).

The opinions of researchers differ in connection with the methods of phenotyping for drought tolerance in wheat. The use of glasshouses allows the precise control of the experimental conditions, such as soil composition, temperature and amount of added water (MAJER et al. 2008; GÁSPÁR et al. 2005; NAGY et al. 2017, 2018). In field trials, however, breeders cannot control the environmental circumstances, as the seasonal water availability for crops differs over the years within the same environment. Thus, the controlled testing of environmental interactions is crucial to obtain reliable results for the selection of improved genotypes (AL-SALIMIYIA et al. 2018).

Heading time is the most critical factor in an ideal adaptation that influences grain yield in environments that vary in water availability and distribution during the growing season (TUBEROSA 2012). Earliness is an important parameter for a breeding programme for drought stress tolerance (LOPES et al. 2012; NAGY et al. 2017, 2018). Several experiments, which applied different levels of water availability on several crops, confirmed the relationship between the plasticity of grain yield and heading time (SADRAS et al. 2009). In this study, all the investigated genotypes under drought stress had earlier heading times than under well-watered conditions, except 'Plainsman V.', which recorded a non-significant slight increase in heading time under drought stress compared to the well-watered conditions. BLUM (2010) demonstrated that a crop's ability to reduce the number of days to heading and the days to maturity could guarantee a drought escape. However, the life cycle of plants should not be too short, in order to avoid grain yield loss

(MWADZINGENI et al. 2016a). The significant correlation between grain yield/plant and heading time under drought stress confirms the results of BENNET et al. (2012) and NAGY et al. (2018) but contradicts the findings of MWADZINGENI et al. (2016a) where the correlation between grain yield/plant and heading time was weak under the same conditions.

Plant height is a simple and appropriate agronomic trait for assessing drought tolerance (ZHANG et al. 2011). Under drought stress, phenotypic changes and the partitioning of dry matter may occur in plants as a response to water deficiency (PASSIOURA 2012). In the current study, the plant height of each studied genotype decreased under drought stress compared to well-watered conditions, with the reduction ranging from 11.00 to 29.40 cm. MWADZINGENI et al. (2016a) confirmed that tall and late-maturing genotypes have the ability and sufficient time to accumulate the photosynthetic assimilates, which result in higher grain yield under well-watered conditions. In our study, the results were in contrast with this finding under well-watered conditions but agreed with it under drought stress. Our results demonstrated that the plant height trait was not in correlation with harvest index under either of the two conditions. This finding was contrary to that of SLAFER et al. (2005), who asserted that reduced plant height was associated with a high harvest index.

In water-limited environments, the pattern of biomass allocation is one of the important adaptive strategies in wheat. Accumulation and allocation of biomass are closely related to the size of plant organs and plant architecture (WANG et al. 2017). Water deficiency negatively affects the biomass production and accumulation of most crops (GROVER et al. 2001). Our results verified that all the studied genotypes, under drought stress, had an average above-ground biomass loss varying between 64.99% and 75.75%. Root dry mass positively correlated with above-ground biomass under well-watered and drought stress conditions. The varieties ‘Plainsman V.’ and ‘GK Berény’ had high above-ground biomass under drought stress, in addition to high root dry mass and grain yield/plant. The capability of these two varieties to absorb water and nutrients was high under drought stress conditions, which is reflected by the above-ground biomass (ELAZAB et al. 2016). A positive correlation was observed between grain yield/plant and above-ground biomass under both conditions. Our findings were harmonious with the results obtained by NAGY et al. (2018).

Selection of genotypes that have a relatively high grain yield under stress and non-stress environments is one of the strategies in plant breeding to improve the adaptation to drought conditions (MWADZINGENI et al. 2016b). Improving grain yield is still in the focus of the breeding programmes (MASON et al. 2013). GAO et al. (2015) reported, however, that there were difficulties in selecting stable high-yielding genotypes under different field conditions, owing to the substantial effect of the environment on grain yield. In the present study, grain yield per plant

decreased in all the investigated genotypes under drought stress compared to the well-watered conditions. The percentages of grain yield reduction ranged between 69.64% and 81.73%. This was attributable to the decrease in above-ground biomass and grain number/plant traits. These results are similar to the findings by NAGY et al. (2018). All the investigated genotypes responded to drought stress with a significant decrease in harvest index, except for the varieties 'Plainsman V.' and 'GK Élet', in which the decrease was not significant. The study by VARGA et al. (2015) verified that harvest index had a significant effect on grain yield. In the current study, no correlation was observed between grain yield/plant and harvest index under drought stress conditions, which supports the results of NAGY et al. (2018) but is contrary to those of VARGA et al. (2015). Our study revealed that the genotypes with high grain yield per plant under both well-watered and drought conditions also had high STI values, which confirms the findings of MWADZINGENI et al. (2016a). The genotypes 'Plainsman V.', 'GK Berény', 'PC61' and 'PC110' had the highest grain yield per plant under both conditions, as well as the best STI values. The obtained results proved the efficiency of the STI index in selection.

The role of root traits in drought tolerance has been fairly well-demonstrated in previous studies (WASAYA et al. 2018), indicating that the effect of water deficiency on plants eventually causes an increase in root growth (KEIM and KRONSTAD 1981). In our study, wheat genotypes responded differently to drought stress for the root length trait. 'GK Berény', 'PC61', 'GK Élet', 'PC92', and 'PC94' achieved increased root length rates varying from 2.36% to 43.96% under drought stress compared to well-watered conditions, while a reduction varying from 1.63% to 12.33% was recorded for this trait in 'Plainsman V.', 'PC110', 'PC332' and 'PC84' under drought stress conditions. The roots play a significant role in the absorption of water and nutrients from deep soil layers during drought stress conditions, and influence the grain yield by their size and architecture, affected by the distribution of soil moisture and the competition levels for water resources within the plant community (KING et al. 2009; WASAYA et al. 2018). Under drought stress conditions, faster-growing genotypes with deeper roots should be used in breeding programmes to guarantee the stability of grain yield, as they can exploit moisture in deep soil layers.

A study by TOMAR et al. (2016) showed that root length correlated positively with both above-ground biomass and grain yield under drought stress conditions, while root dry mass was not in correlation with the grain yield under the same conditions. Various other studies have also emphasised the role of deep and vigorous root systems for increased grain yield in wheat (MANSCHADI et al. 2010; WASSON et al. 2012), barley (FORSTER et al. 2005) and other cereal crops. However, the results in this study were contrary to those of the above-mentioned studies because the root length and root dry mass did not show a correlation with the grain yield under

drought stress. Similar results were obtained in experiments conducted on rice, which revealed a notable lack of correlation between root features and drought tolerance (PANTUWAN et al. 2002; SUBASHRI et al. 2009). NAGY et al. (2018), in their study, also found no correlation between root dry mass and grain yield. The non-correlation between root features and grain yield per plant in the current study may have been due to the use of pots, thereby creating a restriction for deep root penetration. Thus, the large root systems could not be an advantage for the plants. This result resembled the findings of ELAZAB et al. (2016), where there was also restricted root growth in their trial, which was carried out in lysimeters.

On the other hand, the present study revealed a positive correlation between root dry mass and both above-ground biomass and grain number per plant under drought stress. ELAZAB et al. (2016) found a negative correlation between root dry mass and above-ground biomass under a water deficiency regime. Root dry mass reduction was observed in all the investigated genotypes under drought stress compared to well-watered conditions, which the percent reductions ranged between 25.15% and 65.55%. The study of root traits as a selection criterion for drought tolerance faces the difficulty of phenotyping field-grown plant roots (RICHARDS 2008; LEITNER et al. 2014), where the structure and composition of the soil are obstacles in obtaining accurate values for root features in the field study. Therefore, the use of glasshouse pot trials under controlled conditions presents a solution. However, caution is required when applying this type of study, as a lack of quality and quantity of root information can lead to inconsistencies in phenotyping between studies. Furthermore, the study under controlled conditions, in comparison to field conditions, focuses on the effects of a single factor (water regime), while ignoring the interactions between the root system and other environmental factors at the soil level, such as soil type, fertilizer applications, plant density and soil tillage process (ZHANG et al. 2009; SHEN et al. 2013). The study of individual plants grown in glasshouse pots or tubes does not reflect the situation of plants grown in the field.

Overall, the current study demonstrates that selecting drought-tolerant genotypes based on root length and root dry mass traits may be inefficient as a weak correlation between them and grain yield per plant has been found. Further studies on wheat in the field, the growth chambers and the glasshouses using a high number of genotypes to investigate this type of correlation, are required.

6.2. Generation of winter wheat doubled haploid lines via *in vitro* anther culture

Recently, anther culture has been widely used in breeding and applied research, rendering it a highly efficient tool with lower costs than alternative technologies for improving cereals for the development of homogeneity (CASTILLO et al. 2015). Several researchers have reported that albinism and the genotype dependency are limiting factors for the androgenetic production in wheat (JAUHAR et al. 2009; ISLAM and TUTEJA 2012; CHEN et al. 2011; NIU et al. 2014; DWIVEDI et al. 2015). This work shows that these phenomena do not represent major obstacles, where, on average, a similar number of albino and green plantlets was regenerated and, subsequently, doubled haploid lines were produced in all the combinations. Only the green plants were advanced in the breeding programme.

6.2.1. The effect of genotype on anther culture androgenetic production

In the studies carried out by KONDIC-SPIKA et al. (2008), LANTOS et al. (2013), and LANTOS and PAUK (2016), there was no unresponsive wheat plant material without embryo-like structure or green plantlet production, but the inverse was found in the results of HOLME et al. (1999), TUVESSON et al. (2000), BROUGHTON (2008); and EL-HENNAWY et al. (2011). HOLME et al. (1999) concluded that Eastern European wheat was more responsive compared to the North-eastern European genotypes. In our study, the response of plant material to *in vitro* anther culture induction may not have been influenced by the geographical origin (Eastern and Western Europe), as all the combinations produced doubled haploid lines.

The values of embryo-like structures per 100 anthers varied between 6.0 and 74.5, and the maximum value was relatively higher than the values obtained in previous studies: 53% (KIM and BAENZIGER 2005); 52% (KHIABANI et al. 2008); 18% (EL-HENNAWY et al. 2011); and 42% (GRAUDA et al. 2014). The highest embryo-like structures per 100 anthers values exceeding 100% were observed in these studies: 119% (KONDIC-SPIKA et al. 2008); and 169.4% and 190.4% in 2010 and 2011, respectively, (LANTOS et al. 2013).

In the current study, the rate of green plantlets per 100 anthers was 8.3. The values ranging between 0.4 and 5.8 green plantlets/100 anthers were obtained from various previous winter wheat breeding programmes (MASOJC et al. 1993; HOLME et al. 1999; TUVESSON et al. 2000; KONDIC-SPIKA et al. 2008; EL-HENNAWY et al. 2011; LANTOS et al. 2013; GRAUDA et al. 2014). The maximum green plantlets per 100 anthers value was 24.7. Some researchers reported higher than 100 green plantlets/100 anthers in some highly responsive genotypes (BROUGHTON 2011; LANTOS et al. 2013; CASTILLO et al. 2015); others reported maximum values just a bit higher than those obtained in this study (TROTTIER et al. 1993; NAVARRO-ALVAREZ et al. 1994; LANTOS et al. 2013), while others reported less than the current maximum value (KIM and

BAENZIGER 2005; KHIABANI et al. 2008; KONDIC-SPIKA et al. 2008; EL-HENNAWY et al. 2011; GRAUDA et al. 2014). The strategy of TUVESSON et al. (2000, 2003) involved including responsive genotypes in crossing programmes to improve the anther culture efficiency, and led to a reduction of the time to obtain new doubled haploid lines. This highlights the effectiveness of the anther culture method in practical breeding programmes, especially when both green plantlets and doubled haploid lines are produced from every tested combination.

In androgenetic induction via *in vitro* anther culture when plantlets are obtained by primary embryogenesis, the embryo structure is compact, very often having multiple shoot primordia. This means that several shoots are regenerated from one embryo structure, but genetically they are identical plantlets. The trigger of multiple shoot regeneration is the hormone supplement (2,4-D and kinetin) of the medium. In wheat androgenesis induced by anther culture method, primary embryogenesis leads to sufficient regeneration of green plantlet (BROUGHTON 2011; KONDIC-SPIKA et al. 2011; LANTOS et al. 2013; CASTILLO et al. 2015; KANBAR et al. 2020), secondary embryogenesis is deemed unnecessary. The latter is mainly applicable for regeneration in somatic tissue culture. For the haploid system applied in this work, the time between the first microspore division and the regeneration of plantlets is very short, only 5-7 weeks.

6.2.2. Albinism incidence in anther culture

The number of albino plantlets per 100 anthers in this study varied between 0.2 and 22.8 with an overall mean of 5.6, which was low compared to the mean values in previous studies (BROUGHTON 2011; EL-HENNAWY et al. 2011; LANTOS et al. 2013; LANTOS and PAUK 2016). In ten out of the thirteen combinations, low values (0.2–5.8%) of albino plantlets per 100 anthers were recorded, which were not significantly different at $P < 0.05$. This finding is in contrast to the results obtained by WĘDZONY et al. (2009) and JAUHAR et al. (2009), who indicated that albinism hindered the androgenetic doubled haploid production in some genotypes. Similarly to the findings presented by TUVESSON et al. (2000); KONDIC-SPIKA et al. (2011); LANTOS and PAUK (2016), the effect of genotype (combination) was significant for all the studied androgenetic traits.

The rate of albino regenerants varies within the same species, and some genotypes have a higher response than others (MAKOWSKA and OLESZCZUK 2014; KRZEWSKA et al. 2015). In our study, the low frequency of albino regeneration could be attributed to the genetic material being of Eastern Europe origin or to the interaction between the combination and the cold-pre-treatment of tillers, which was at 3–4°C for 18 h light for 2 weeks. In anther culture, the increase of the androgenetic efficiency can be obtained by applying sufficiently strong stress that leads to the alteration of the microspore development pathway. Identifying the appropriate pre-treatment

is needed for androgenetic efficiency, and should be suitable in order not to lead to high mortality of cells or disrupt cellular function (MAKOWSKA and OLESZCZUK, 2014). LAZARIDOU et al. (2016) revealed that cold-pre-treatment of hexaploid wheat spikes for 18 days influenced positively the AC response in some genotypes and negatively in others, and was better than the pre-treatment of spikes for 7 days when using W_{14} and 190-2 as the induction and regeneration medium, respectively. Nevertheless, RIZKALLA et al. (2012) found that cold-pre-treatment of wheat spikes for 7 and 14 days had almost the same effect on embryo-like structure induction.
The incubation temperature was 28°C. The convenient temperature range is between 28 and 30°C as OUYANG et al. (1983) reported for cereal crops; higher incubation temperatures can lead to an increased frequency of albino plantlets.

The number of albinos among the regenerants could also be affected by the components of the induction or the regeneration medium. That was demonstrated by many studies, which showed the role of the copper element (JACQUARD et al. 2009), the polyamine treatments (REDHA and SULEMAN 2011), and the *n*-butanol treatment (SORIANO et al. 2008; BROUGHTON 2011) in increasing the ratio of green plantlets to albino plantlets in anther culture. In the experiment conducted by WEIGT et al. (2020) to compare the androgenetic response of fifteen spring and fifteen winter wheat genotypes by using C17 induction medium supplemented by two combinations of growth hormones [the only auxins (2,4-D and dicamba), and auxin with cytokinin (2,4-D and kinetin)], the results showed that the spring genotypes were higher responsive considering embryo-like structures and green plantlets in each medium, compared with the winter ones. In addition, the spring wheats achieved higher androgenetic production of embryo-like structures and green plantlets by using C17 induction medium with only auxins compared to the C17 induction medium with auxin and cytokinin hormones. On the contrary, the winter wheats produced more androgenetic production of embryo-like structures and green plantlets on the C17 induction medium with auxin and cytokinin hormones compared to the other medium, which proves that the selection of appropriate composition of the medium is crucial for increasing the efficiency in anther culture. Albino plantlets are formed when proplastides cannot transform into chloroplasts (MAKOWSKA and OLESZCZUK 2014). The main causes of this incidence are still ambiguous, but genetic components, as reported by (VAUGHN et al. 1980; DAY and ELLIS 1985; ANKELE et al. 2005) may overlap to interpret it. Studying the effect of the genotype with more improvements in the pre-treatments or the component of the induction and regeneration medium can contribute to overcome or to reduce albinism incidence, thus increasing the anther culture efficiency.

6.2.3. *In vivo* acclimatization of plantlets

After acclimatisation, the frequency of plantlets per 100 transplanted plantlets varied between 36.4% and 98.0% depending on the combination. The plantlet losses originated from the hardening of the plantlets, such as transferring them gradually from high to low humidity and from low to high light intensity, besides the transplantation itself. These losses can be overcome by applying a suitable concentration of sucrose (2–4%) or growth retardants to the shoot and root induction media, and also reducing the moisture in the culture boxes by adding oily substances that subsequently improve the growth of the plants *in vivo*. Although experiments to optimise the conditions for plantlet micropropagation *in vivo* were not fully presented, studies about improvements of the conditions of *in vitro* plantlet micropropagation of different plants were conducted to improve the plantlet acclimatisation subsequently in the greenhouse (POSPISILOVA et al. 1999; HAZARIKA 2003). Furthermore, the transfer of plantlets from the laboratory to the greenhouse or to the field (nursery) is a critical point of this work. Increasing the *in vivo* plantlet frequency depends significantly on the improvements of the *in vitro* shoot and root induction media, genetic effects, the human background, and the technical equipment. Among the regenerated plantlets, there are a few percent (5–10 %, depends on the season) of the chimeric and genetically changed individuals (mono-some, tri-some, etc.), which need special care. These individuals are not beneficial for breeding programmes. As a rule, they degenerate and do not survive the greenhouse- or field (nursery) breeding circumstances.

6.2.4. Doubled haploid production

The overall ratio of doubled haploid plants per 100 acclimatised plantlets lines in the present investigation was higher than those reported by KIM and BAENZIGER (2005), KONDIC-SPIKA et al. (2008), LANTOS et al. (2013), LANTOS and PAUK (2016), who obtained 49%, 47.9%, 35%, and 32.7%, respectively. Spontaneous doubled haploid wheat lines, which varied between 5 and 30%, were found in the studies conducted by ZIEGLER et al. (1990); MASOJC et al. (1993); and NAVARRO-ALVAREZ et al. (1994). In our experiment, three combinations showed higher doubled haploid plants/100 acclimatised plantlets rates (76.6%, 87.1%, and 87.8%) than the average, indicating that this parameter is a remarkable tool for selecting responsive genotypes to integrate them into cross-breeding programmes and thus achieve increased anther culture efficiency.

Overall, the androgenetic production of wheat via anther culture is affected by albinism (WĘDZONY et al. 2009; BROUGHTON 2011) and genotype dependency (ISLAM and TUTEJA 2012; DWIVEDI et al. 2015), which were mitigated in this investigation, but other factors associated with laboratory manual work, physical factors (i.e., light, temperature) (ISLAM and

TUTEJA 2012) and non-controlled factors could overlap in reducing the androgenetic production induced by anther culture. Therefore, excluding the obstacles of wheat anther culture method is a needed task to obtain satisfying results for researchers.

7. CONCLUSIONS AND RECOMMENDATIONS

7.1. Characterization of winter wheat genotypes for drought tolerance

- The irrigation system used in this investigation can be efficiently applied to evaluate and select drought-tolerant genotypes in breeding programmes.
- Each genotype showed a decrease in all the studied traits under water deficiency compared to well-watered conditions.
- Each investigated genotype had grain yield loss under drought stress conditions. According to their grain yield reduction and STI values, 'Plainsman V.', 'GK Berény', and 'PC61' had the highest drought tolerance among the tested genotypes.
- A positive significant correlation was recorded between the traits grain yield/plant and grain number/plant under both well-watered and drought stress conditions.
- This study revealed that the selection for high above-ground biomass results in selection for high grain yield per plant under both conditions.
- Our results pointed out the importance of the genotypes having high above-ground biomass and grain number per plant for increasing grain yield under drought stress.
- It was also shown that the genotypes with higher amount of root dry mass have higher amount of above-ground biomass under drought stress.

7.2. Generation of winter wheat doubled haploid lines via *in vitro* anther culture

- This investigation showed the importance of *in vitro* haploid induction via anther culture in a winter wheat breeding programme.
- Each crossing combination produced green plantlets and doubled haploid lines in a sufficient number.
- The albinism incidence was found in each combination.
- Although the fluctuation of the anther culture was present in each studied parameter, the genotype dependency was not the hindering factor.
- The combinations 'Béres/Midas', 'Kalász/Tacitus', 'Béres/Pamier', and 'Premio/5009' achieved the highest rates of the doubled haploid production.
- The above-mentioned doubled haploid lines are recommended as effective basic genetic materials in crossing programmes for increasing the numbers of doubled haploid plants in consequent experiments.
- The total number (923) of the generated doubled haploid lines will be included in different wheat drought-tolerance experiments for releasing improved candidates.

8. NEW SCIENTIFIC RESULTS

- In the present study, we confirmed the previous results of drought-tolerant selected genotypes from the field experiment by using the controlled assessment of environmental interactions.
- The use of this type of study in the glasshouse enabled the easily-phenotyping of the root traits as a selection criterion for drought tolerance while phenotyping the field-grown plant roots presents difficulty.
- We showed that the 'Plainsman V.', 'GK Berény, and 'PC61' genotypes are the most drought-resistant and high-yielding under stress conditions.
- By modifying the anther culture protocol of winter wheat (*Triticum eastivum* L.), green plantlets were produced in all genotypes and we improved the green plantlet production.
- We significantly increased the doubled haploid, including spontaneous doubled haploid production (87.8, 87.1, and 76.6%), and the doubled haploid lines have been generated in all the studied combinations for the breeding programmes.
- Albinism and genotype dependency – limiting-factors for wheat doubled haploid production induced by *in vitro* anther culture – were mitigated by the application of anther culture method in this study.
- Doubled haploid lines with modified anther culture have been developed for breeding programmes to make plants endure drought better.

9. SUMMARY

Climate change realities such as high temperature are among the causes of drought episodes affecting the productivity and yield stability of crops worldwide. Breeders, therefore, face a daunting challenge to overcome a large gap in the agricultural sector arising due to drought through the improvement of new tolerant genotypes. These genotypes involved in breeding programmes for drought tolerance evaluation could be produced by applying doubled haploid technology, which enables the development of genetically homozygous pure lines from heterozygous breeding material in one generation, thus it is a fast alternative to the conventional breeding methods and has become an indispensable method in the attainment of homogeneity in different researches and breeding programmes.

The present study, consisting of two experiments, was performed during 2018–2020. The first study was executed to characterise winter wheat doubled haploid lines for drought tolerance under well-watered and drought stress conditions in the glasshouse, while the second one was carried out to generate winter wheat (*Triticum aestivum* L.) doubled haploid lines using *in vitro* anther culture.

For this purpose, the first study included nine winter wheat genotypes (three varieties and six doubled haploid lines selected based on the study of NAGY (2019) [drought-tolerant (PC61, PC110, and PC332) and drought-sensitive (PC84, PC92, and PC94)], and was carried out to assess the performance of these genotypes under well-watered and drought stress conditions for the traits heading time, plant height, above-ground biomass, main spike length, spikelet number per plant, fertile spikelet number/plant, grain number/plant, grain yield/plant, harvest index, 1000-grain weight, root length, and root dry mass. While the second study investigated the anther culture efficiency in thirteen F_4 combinations of winter wheat (*Triticum aestivum* L.). The genotype dependency was evaluated during the induction of embryo-like structures as well as green and albino plantlets and during the transplantation of the regenerated plantlets. The frequency of the spontaneous doubled haploids was also assessed.

In the first study, lower grain yield per plant values were observed for each investigated genotype under drought stress than under well-watered conditions. The percent reduction of grain yield per plant varied between 69.64% and 81.73% depending on the genotype. The correlations between the grain yield per plant and heading time, above-ground biomass, and grain number per plant were positive and significant under the drought stress. The genotypes having high root dry mass values showed both high above-ground biomass and grain number per plant values under drought stress. Grain yield/plant reduction had positive correlations with plant height, grain number per plant, and harvest index reductions. The second study revealed that each crossing combination produced embryo-like structures, as well as green and albino plantlets. After

acclimatisation, the green plants were transplanted in the nursery and spontaneous doubled haploid grains were harvested from the transplanted individuals. The number of embryo-like structure per 100 anthers varied from 6.0 to 74.5, with the overall mean of 35.8. The number of green plantlets per 100 anthers ranged between 0.4 and 24.7 with an average of 8.3. Albino regenerantes occurred in each crossing combination. Depending on the combination, the value of albino plantlets per 100 anthers ranged between 0.2 and 22.8 with an average value of 5.6. The value of doubled haploid plants were also produced in each combination. The value of doubled haploids per acclimatised plantlets varied between 25.0 and 87.8 with an average of 59.7.

In the first study, each genotype recorded grain yield under drought stress, and the varieties 'Plainsman V.', 'GK Berény' and the doubled haploid lines 'PC61', 'PC110' showed the best drought tolerance. These genotypes will be involved in various drought tolerance trials and breeding programmes. As regards the second study, the combinations: 'Béres/Midas', 'Kalász/Tacitus', 'Béres/Pamier', and 'Premio/5009' had the highest doubled haploid production. This contributes remarkably to the selection of the most appropriate genetic materials in the subsequent cross-breeding programmes. Our observations highlight the usability and efficiency of *in vitro* anther culture in the production of a large number of doubled haploid lines for the breeding and the applied researches of winter wheat. Although albinism was shown in each combination, it was mitigated by using our *in vitro* anther culture protocol.

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