The Thesis of the PhD dissertation

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Szeged-Gödöllő

2021

Hungarian University of Agriculture and Life Sciences

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

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2021

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1. BACKGROUND OF THE WORK

1.1. Characterization of winter wheat genotypes for drought tolerance

Common wheat (*Triticum aestivum* L.) growing in different environments is one of the main strategic cereal crops in the world. Wheat is a key component of global food security and provides 20% of the total calories consumed worldwide (NAGY et al., 2018). 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 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).

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 (NAGY et al. 2018). Knowledge of the phenotype response of plants is urgently needed in breeding programmes to release highly productive with 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).

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).

The roots are characterised by a spectacular level of morphological plasticity in response to the physical soil conditions (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).

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).

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. 2016).

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

In vitro anther culture is one of the efficient biotechnology methods in plant breeding of wheat to produce doubled haploid lines from microspores in anthers. The technology 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) including quantitative trait loci (QTLs) (SHI et al. 2019).

The main methods applied in breeding to produce doubled haploid lines involve wide hybridisation, gynogenesis, and androgenesis (DUNWELL 2010). 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 (IMMONEN and TENHOLA-ROININEN 2003; DUNWELL 2010).

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

Genotype dependency is the main obstacle to doubled haploid wheat production via *in vitro* anther culture (ISLAM 2010; BROUGHTON 2011; LANTOS et al. 2013). The response of wheat to androgenetic induction by anther culture differs depending on the genotype, among species, and even within species.

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.

Several kinds of research have been conducted in recent decades to improve the efficiency of wheat anther culture by crossing with responsive genotypes (TUVESSON et al. 2000, 2003). In addition, 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 chemicals) 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 (BROUGHTON et al. 2020; WEIGT et al. 2020).

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} (LANTOS et al. 2013), and P_2 (KONDIC-SPIKA et al. 2011). There are other induction media, too, such as C17, LIM, MS3M and AM.

Spontaneously doubled haploid is commonly shown among cereal plants produced by anther culture. It is a safe process because colchicine has a toxic effect to humans.

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 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 involved 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 (see Materials and Methods) 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. MATERIALS AND METHODS

3.1. Characterization of winter wheat genotypes for drought tolerance

3.1.1. Plant material and cultivation method

This study included nine wheat genotypes: six pre-selected DH 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.

3.1.2. 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). Each genotype per treatment was given the same amount of water each time (twice a week) with the average irrigation requirement of the plants. 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.

3.1.3. Investigated traits

Before harvesting, the morphological traits were recorded, such as days to heading, plant height. 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 per plant, fertile spikelet number per lant, grain number per plant, grain yield per 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, before being dried at 25–30^{o}C in the shade, after which the root dry mass was estimated.

3.1.4. Experimental design and statistical analysis

The experiment was conducted in a randomised complete block design with well-watered and drought stress treatments and five replications, 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). 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 genotype and treatment, F value, and F probabilities for all the tested traits. The correlation matrix was generatedusing Pearson product-moment correlation and pairwise-P values to calculate the significance of correlation coefficient values. The fitted linear regression model was used to examine the relationship between the traits. Stress tolerance index (STI) was calculated according to FERNANDEZ (1992).

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

3.2.1. Plant materials

Thirteen F_4 combinations (accessions) (‘Sel.9/DH150’, ‘Premio/5009’, ‘DL41/DH150’, ‘DL45/DH150’, ‘Béres/Midas’, ‘Béres/Pamier’, ‘Kalász/Tacitus’, ‘Kolo/Premio’, ‘Körös/Premio’, ‘Midas/Csillag//Tacitus/5003’, ‘DH54/12.189’, ‘DH54/12.89’, ‘Kapos/Ködmön’) were selected for this study from the drought tolerance trial of thirteen winter wheat F_3 plant materials provided by the Cereal Research Non-profit Ltd. (CR Ltd.). The agricultural practices of the wheat crop were applied from fertilisation to pest control depending on the standard protocol for small grain winter cereals (LANTOS et al. 2013).

3.2.2. 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 the nursery. Donor tillers were then 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.

3.2.3. 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) were sterilized under a flow box. 300 anthers per replication were isolated by fine forceps and put onto a 90 mm plastic Petri dish (Sarstedt, Budapest, Hungary) containing 15 mL of a liquid W14mf induction medium.

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. 10 replications per genotypes were prepared.

3.2.4. Plantlet regeneration

About 5-weeks after the incubation, approximately 30–35 embryo-like structures with a diameter of 1–2 mm 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) and put in a lighted growth room. After about 2–3 weeks, approximately 15–18 of the green plantlets with a length of 20–30 mm, were transferred into 1000 mL plastic boxes filled with a solid regeneration medium. The boxes were kept in a growth room (24^{o}C, 16/8 h light/dark photoperiod, fluorescent light at 200 µmol/m^2/s) for the regeneration of whole plantlets. The albino plantlets were counted and discarded.

3.2.5. 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 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. In October, the plants in the cold chamber were transplanted to the ‘doubled haploid nursery’. During the growing season in autumn and winter, many different stresses (cold, frost, short days etc.) affect the plants restoring the fertility. 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.

3.2.6. 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.

4. RESULTS

4.1. Characterization of winter wheat genotypes for drought tolerance

4.1.1. The response of the studied traits to water deficit

High significant differences of genotype and treatment effects were recorded in all traits except root length. Significant differences at $P < 0.001$ probability level were obtained in the heading time and plant height traits, and at $P < 0.01$ in the main spike length and 1000-grain weight traits, while significant differences at $P < 0.05$ were recorded in the traits of above-ground biomass, grain number per 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 per plant, fertile spikelet number per plant and grain yield per plant.

In this investigation, the influence of water deficiency on wheat genotypes was observed on all the studied traits.

4.1.1.1. 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.

4.1.1.2. Plant height

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 rates of plant height

reduction under drought stress conditions ranged between 14.56% in ‘Plainaman V.’ and 36.66% in ‘PC332’.

4.1.1.3. Above-ground biomass

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 percent reduction of above-ground biomass caused by drought stress ranged from 64.99% to 75.75%.

4.1.1.4. Grain number per plant

Water deficiency caused a significant drop in the grain number per plant of each investigated genotype; ‘PC84’ had the smallest difference in 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 percent reductions of the grain number per plant of ~~all~~ the genotypes varied from 64.84% to 79.01% under drought stress compared to well-watered conditions.

4.1.1.5. Grain yield per plant

The grain yield per plant of each investigated genotype decreased significantly under drought stress compared with the well-watered conditions. The values of grain yield per 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 reduction percentage was from 69.64% to 81.73%. The genotypes ‘Plainsman V.’, ‘GK Berény’ and ‘PC110’ had the best performance of grain yield per plant according to their reduction index being the lowest among all values (69.64, 76.51 and 77.08%, respectively). The calculated STI of the genotypes was from 0.298 to 0.179. The highest values of STI were observed in ‘Plainsman V.’, ‘GK Berény’, and ‘PC61’ (0.298, 0.261, and 0.214, respectively).

4.1.1.6. 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. Under drought stress, only four

genotypes ‘PC84’, ‘Plainsman V.’, ‘PC110’ and ‘PC332’ showed percent reduction of the root length (1.63, 2.31, 8.28 and 12.33%, respectively).

4.1.1.7. Root dry mass

A significant decrease was observed in the root dry mass trait of most genotypes under drought stress. 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.’. The loss percentages of root dry mass caused by drought stress ranged between 25.15% and 65.55%.

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

Heading time correlated significantly with grain yield per plant and plant height under drought stress. Grain yield per plant showed a positive correlation with grain number per plant under both conditions, while root dry mass correlated positively with grain number per plant under drought stress. Grain yield per 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 per plant reduction and plant height, fertile spikelet number per plant, grain number per plant and harvest index reductions.

4.2. 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.

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

The number of the embryo-like structures per 100 anthers varied between 6.0 and 74.5, depending on the combination (genotype). The overall mean of the 13 F_4 combinations was 35.8 embryo-like structures/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’ ~~one~~. 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). The overall mean of the combinations was 8.3 green plantlets/100 anthers. Albino plantlets were found in each combination. The values per 100 anthers were between 0.2 and 22.8. The overall mean value was 5.6 albino plantlets/100 anthers.

4.2.2. Production of doubled haploid lines

A total of 1545 acclimatised plantlets were obtained in this experiment. 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)**.**

5. CONCLUSIONS AND RECOMMENDATIONS

5.1. 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.

5.2. 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 involved in different wheat drought-tolerance experiments for releasing improved candidates.

6. NEW SCIENTIFIC RESULTS

- In the present study, we confirmed the previous results of drought-tolerant selected genotypes by using the controlled assessment of environmental interactions.
- The use of this type of study in the glasshouse enabled the easily-phenotyping 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.

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