HUNGARIAN UNIVERSITY OF
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Haploid Breeding of Triticale and Its Novel Utilization

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1. BACKGROUND AND OBJECTIVES

This PhD dissertation reports results from two key fielkds of research: 1. the use of haploid methods in triticale breeding (Jr. Kruppa et al. 2023) and 2. the utilization of triticale as a forage crop (Jr. Kruppa et al. 2025). Both topics are important not only for practical breeding applications but also from a research perspective. Although the haploid field has a longer literature history, there are relatively few references to routine breeding applications. Our focus on triticale for forage was motivated by global climate change and the crop's excellent nutritive value.

One major aim was to compare the efficiency of two commonly used basal media (P4 and W14) in anther culture of triticale cultivars and their F_1 hybrids. We evaluated the effects of medium, genotype, and the genotype × medium interaction on triticale androgenesis, using the following androgenic response traits: number of embryo-like structures (ELS), number of green and albino regenerants, and number of acclimatized/transplanted plantlets. We also verified the homogeneity (homozygosity) of the resulting doubled haploid (DH) lines in the DH_2 generation with SSR (simple sequence repeat) markers, in order to assess the feasibility of intercrossing (cross-pollinating) the produced DH triticale lines.

In modern agriculture, small-grain cereals have a wide range of uses. Beyond the traditional focus on grain yield, the utilization of green biomass has gained remarkable importance in recent years. Maize silage remains the cornerstone roughage for ruminants - especially dairy cattle - yet its cultivation faces growing challenges under climate change. In our region, maize flowering and grain filling often coincide with the driest, most rainfall-deficient period of the

growing season, which can lead to substantial yield losses, premature harvest, and quality deterioration. Consequently, production systems that better exploit winter-season precipitation and provide high-quality forage with adequate green yield have come to the fore. Alongside food uses of triticale, Kruppa-Mag Ltd. - in cooperation with the Hungarian University of Agriculture and Life Sciences (MATE), Kaposvár and Szent István Campuses, and Livestock Performance Testing Ltd., Gödöllő (Állattenyésztési Teljesítményvizsgáló Kft.) - was the first in Hungary to initiate systematic studies on triticale as a forage crop. These efforts focused on green yield and the nutritive (proximate and fiber) composition of the biomass.

Based on the above, our main objectives were:

1. To compare the P4 and W14 induction media for the anther culture of triticale cultivars and their F_1 hybrids.

2. To assess the effects of medium, genotype, and their genotype × medium interaction on triticale androgenesis under *in vitro* anther culture.

3. To verify the genetic homogeneity (homozygosity) of the produced triticale doubled haploid (DH) lines in the DH_2 generation using SSR molecular markers.

4. To evaluate triticale as a green forage from quantitative (yield) and qualitative (nutritive value) perspectives in a set of cultivars currently under production.

2. MATERIALS AND METHODS

2.1. Production of haploid plants and homogeneity testing

2.1.1 Plant material and cultivation conditions

We used four Hungarian-bred triticale cultivars (’Hungaro’ = H, ’GK Szemes’ = SZ, ’Dimenzio’ = D, ’GK Rege’ = R) and eight F_1 hybrids (’H×R’, ’H×SZ’, ’H×D’, ’SZ×H’, ’SZ×D’, ’D×H’, ’D×R’, ’D×SZ’) as donor genotypes for the anther-culture experiments. The selected genotypes were sown in the breeding nursery of Cereal Rresearch Non-Profit Ltd. (Szeged, Hungary) and grown following standard European protocols. Fertilization comprised an autumn application of NPK (1:1:1) at 12 g/m^2, supplemented in mid-April with ammonium nitrate at 18 g/m^2. Insect pests were controlled with two insecticide treatments. Weeds were removed mechanically before sowing, followed during the growing season by chemical control and manual weeding.

2.1.2 Pretreatment of donor spikes

The developmental stage of microspores was monitored with an Olympus CK-2 inverted microscope (Olympus, Southend-on-Sea, UK). Donor spikes containing microspores at the early to mid uninucleate, vacuolated stage were collected. To induce *in vitro* androgenesis, a cold pretreatment was applied: donor spikes were placed in 300 ml Erlenmeyer flasks filled with tap water, covered with PVC bags, and kept in a cold room for two weeks at 2 - 4°C under continuous low-light (semi-dark) conditions.

2.1.3 Anther isolation and *in vitro* anther culture

Prior to isolation, the developmental stage of the microspores was re-evaluated using an Olympus CK-2 inverted microscope (Olympus, Southend-on-Sea, UK). From spikes containing uninucleate, vacuolated microspores, anthers were isolated with ophthalmic forceps and used to initiate in vitro anther cultures. Before isolation, the spikes were placed in 300 ml of 2% NaO'Cl solution containing one drop of Tween-80 and shaken for 20 min for surface sterilization. After sterilization, donor spikes were rinsed three times with sterile distilled water. Anthers from the donor genotypes were plated into 55 mm plastic Petri dishes (Sarstedt Ltd., Newton, MA, USA) at 100 anthers per dish, with four replicates per treatment. Each dish contained one of two induction media, W14mf (Ouyang et al. 1989; Lantos et al. 2013, 2014) or P4mf (Ouyang et al. 1973; Pauk et al. 2003b). During the first 3 days, cultures received a heat shock at 32°C in the dark, then were incubated in a dark thermostat at 28°C until the appearance of embryo-like structures (ELS) and their transfer to regeneration medium. The emergence of microspore-derived ELS was monitored weekly, and newly formed ELS were transferred to regeneration medium.

2.1.4 Plant regeneration and acclimatization

Microspore-derived ELS (≤ 2 mm) were transferred to 90-mm plastic Petri dishes (Sarstedt, Newton, MA, USA) containing '190-2 Cu' regeneration medium (Pauk et al. 2003a). Within 2–3 weeks, green and albino plantlets regenerated. Green plantlets were transferred individually into glass tubes for rooting, using the same regeneration medium. Well-rooted green plantlets were potted in the greenhouse into plastic containers filled with a 1:1 ration of peat- and sand soil mixture. During the 3-5 day acclimation period, plantlets

were covered with PVC. Acclimatized plants were grown to maturity in a climate-controlled greenhouse (20–25 °C). In autumn, green plantlets were hand-transplanted to the DH nursery, and DH_0 plants were cultivated to harvest following the protocol described in Section 2.1.1. The percentage of spontaneous chromosome doubling was calculated after harvesting fertile spikes of transplanted DH_0 plants as: (number of fertile plants / number of transplanted plants) × 100. In the DH_1 generation, DH lines were further propagated in the nursery according to the standard protocol (Section 2.1.1).

2.1.5 Primers

Xwmc primers were originally developed for hexaploid wheat by the Wheat Microsatellite Consortium (WMC) (Song et al. 2002). SCM (*Secale cereale* microsatellite) primers were designed on the basis of the rye genome (Rychlik and Rhoads 1989), while BARC primers originated from a U.S. barley–wheat mapping program (Song et al. 2002). Molecular marker–based genotyping of triticale cultivars was first initiated by leveraging the results of comparative mapping (Van Deynze et al. 1998). Following the observations of Kuleung et al. (2004) - who demonstrated that 58% of 182 wheat markers and 39% of 28 rye markers were suitable for triticale cultivar analysis - we likewise employed these wheat (Xwmc/BARC) and rye (SCM) primers.

2.1.6 DNA isolation, PCR, microsatellite analysis, and allele sizing

For the molecular genetic analyses, leaf samples were collected from two cultivars – ’Hungaro’ (46 DH lines) and ’GK Rege’ (R, 10 DH lines) – and from six hybrids: ’R×H’ (5 DH lines), ’H×R’ (32 DH lines), ’D×R’ (12 DH lines), ’D×H’ (10 DH lines),

’HxD’ (3 DH lines) and ’R×D ’(3 DH lines) all in the DH_2 generation. DNA extractions were performed using the DNeasy® Plant Mini Kit according to the manufacturer’s instructions (QIAGEN, Hilden, Germany).

2.2. Evaluation of triticale as a green forage and its nutritive composition

Between 2017 and 2020 at Szarvas, we examined grain yield performance, green forage yield, and the key nutritive composition parameters of triticale green biomass. The field trial was established along the Orosházi main road on the premises of Mezőmag Ltd. The soil is a clay loam with an acidic to slightly acidic reaction; the cultivated layer contains no $CaCO_3$. Based on humus content, the soil’s N-supplying capacity was medium. Soil water relations are characterized by moderate hydraulic conductivity and high water-holding capacity. The tilled layer is compacted, with lower total porosity and a reduced proportion of gravitational pores.

The triticale cultivars (genotypes) tested were ’Hungaro’, ’Dimenzio’, ’GK Szemes’, and ’GK Maros’. In all four years we recorded grain yield and green yield. At each cutting, green biomass and dry matter (DM) content were measured, and forage nutritive value was determined from the samples: crude protein, crude fiber, aNDFom, dNDF, $NDFd_{48}$, NEI, lysine, and methionine. Cutting dates were scheduled to track the dynamics of both yield and forage quality/nutrient composition. To ensure comparability among cultivars for green yield, results were standardized to a common DM basis by converting cutting yields to 30% DM. Identical agrotechnical management was applied in every year of the trial.

Green biomass measurements – carried out for the first time in Hungary – were performed by harvesting triticale varieties at different phenological stages, using the BBCH scale. Each year, at every harvest date, four 1 m² samples were taken from each variety. The first harvest was conducted when the spike was approximately 6 cm long and had not yet emerged ("booting stage"), corresponding to BBCH 45. Subsequent harvests were performed at 7-day intervals. The developmental stages were as follows: second harvest at BBCH 49 (first awns visible), third harvest at BBCH 58 (80% of spikes emerged), and fourth harvest at BBCH 65 (full flowering). The harvested green biomass was chopped into 2–5 cm pieces, and subsamples were collected and analyzed at the Feed Analysis Laboratory of the Animal Breeding Performance Testing Ltd. in Gödöllő. Based on the dry matter content of the harvested samples, green biomass yields were uniformly corrected to a standardized dry matter content of 30%. In addition to green biomass, we also evaluated the grain yield of the triticale varieties. Harvesting was carried out using a combine harvester at full maturity (BBCH 89).

Forage quality analyses:

We determined the following parameters:

- Moisture, according to MSZ ISO 6496:1993.
- Crude protein (CP), according to NEN-ISO 5983-2.
- Crude fiber (CF), according to NEN-EN-ISO 6865.
- aNDFom, ADF, ADL, according to NEN-EN-ISO 13906, Van Soest (1963) methodology.
- $NDFd_{48}$ (*in vitro* 48 h incubation in rumen fluid).

- NEI (net energy for lactation) and ME (metabolizable energy), according to the German protein and energy evaluation system (GfE).

2.3. Statistical analyses

In the in vitro experiments, each treatment was replicated at least four times. Data from anther culture (ELS, numbers of green and albino regenerants, and transplanted plantlets) were recorded throughout the experiments. The androgenic response variables were analyzed by two-factor ANOVA. The regeneration rate (%) = (number of regenerated plantlets / number of ELS) × 100 and the green regeneration (%) = (number of green plantlets / number of ELS) × 100 were evaluated using two-factor ANOVA without replication. These analyses were performed with Microsoft Excel 2013 (Microsoft, Redmond, WA, USA).

For the forage trials, comprehensive analytical testing was carried out on all harvested green-yield samples, and results were evaluated across cutting dates and cultivars. The statistical evaluation of field results was conducted in SPSS for Windows 25.0, including analysis of variance (ANOVA), correlation and regression analyses.

3. RESULTS

3.1. Generation of doubled haploid (DH) plants and homogeneity assessment

In cereal breeding, in vitro androgenesis-based methods are frequently used for the production of doubled haploid (DH) plants. The aim of the present study was to determine the effect of genotype (four varieties and eight F1 hybrids) and induction medium (W14mf and P4mf) in triticale (X *Triticosecale* Wittmack) anther culture. Androgenesis was successfully induced in all examined genotypes and treatments. The efficiency of anther culture was significantly influenced by genotype, which was evaluated based on the following parameters: number of ELSs, albino plantlets, and green plantlets. Consistent with previous reports (Eudes and Amundsen, 2005; Gonzalez et al. 2005; Zur et al. 2008; Lantos et al. 2014), genotype significantly affected the numbers of ELS ($p \leq 0.001$), albino plantlets ($p = 0.01$), green plantlets ($p \leq 0.001$), and transplanted plantlets ($p \leq 0.001$).

The induction medium also had a significant effect on the number of ELSs, albino plantlets, and transplanted plantlets. Two media (P4mf and W14mf) were used in the triticale anther culture experiments. The efficiency of anther culture was higher on the P4mf medium (103.7 ELS/100 anthers, 19.7 green plantlets/100 anthers) compared with the W14mf induction medium (90.0 ELS/100 anthers, 17.0 green plantlets/100 anthers). In the plant regeneration phase, ELSs derived from the W14mf medium showed a higher regeneration percentage of green plantlets (18.0%), whereas those obtained from the P4mf medium exhibited a lower regeneration percentage (15.9%). The spontaneous chromosome-doubling rate is generally low in

triticale anther culture; most reports cite values below 35% (Arzany and Darvey 2001; Würschum et al. 2012; Lantos et al. 2014). In our experiment, the average chromosome-doubling rate across the genotypes tested was 29.28%.

After growing the DH_0 plants and obtaining seeds, the DH_1 generation was raised, and the genetic homogeneity of the subsequent DH_2 progeny was assessed using molecular genetic methods. Most of the examined DH lines were characterized by homogeneity, and following agronomic selection, they were integrated into the breeding program. Although the doubled haploid (DH) technique provides an excellent opportunity for producing genetically uniform lines, our observations revealed that a small proportion of DH lines (2–8%) exhibited heterogeneity. We attribute this to the exceptionally high outcrossing tendency of triticale, which considerably exceeds the outcrossing rates observed in wheat. This phenomenon may pose a serious risk during breeding and variety maintenance processes if propagation is not carried out under properly isolated conditions.

3.2. Utilization of triticale as a green forage

Between 2017 and 2020, we evaluated the grain yield performance, green biomass production, and key nutritional parameters of different triticale varieties in field trials conducted at Szarvas, Hungary.

The average grain yields of the triticale varieties ranged from 4.39 to 5.22 t/ha. Statistical analysis did not confirm significant differences among the yield averages of the varieties. Although grain yield was not the primary focus of our experiment, we established that the results were fully consistent with the site averages, indicating that

the performance of the triticale varieties corresponded to the long-term regional mean.

Across 2017–2020, the triticale genotypes produced 18.836–31.555 t/ha green biomass, which aligns with published ranges (Sharma 2023, 15,2–21,4 t/ha; Cui et al. 2025, 20,1–29,8 t/ha). In the evaluation of green biomass yields, we found that the varieties did not follow the same growth dynamics. In every year of the study, the green biomass increased with the progression of the harvest dates; however, the rate and pattern of this increase varied considerably among the varieties. Our results indicate that green yield is strongly influenced by the growing season rather than being a consistent varietal characteristic. Consequently, statistically verifiable differences in green biomass among the varieties could not be confirmed during the study years.

In the evaluation of the nutritive value of green biomass, it was observed that the crude protein content of triticale decreased continuously and significantly with successive harvests, a pattern corroborated by the international literature (De Zutter et al. 2023). On average across varieties, the crude protein content measured at the first harvest (BBCH 45) was 165.75 g/kg DM, which declined to 101.82 g/kg DM by the fourth harvest (BBCH 65). Significant differences were also found among varieties: the crude protein content of the variety 'GK Szemes' averaged only 111.34 g/kg DM, compared to 123.33–128.29 g/kg DM for the other varieties.

Another important indicator of green biomass quality is the crude fiber and aNDFom content, both of which were monitored in terms of quantitative levels and qualitative changes affecting digestibility. In the experiment, the highest crude fiber concentration across the four years and 16 harvest dates was recorded 11 times in

the variety ’GK Szemes’, which was also associated with a lower crude protein content. Thus, it can be concluded that the forage value of „GK Szemes” is weaker. The crude fiber content of the varieties was significantly lower at the first harvest (BBCH 45; 238.38 g/kg DM), and it increased up to the third harvest (BBCH 58; 288.63 g/kg DM), where it reached a significantly higher level compared to the other sampling dates.

Statistically significant differences in dNDF content were observed across the different harvest dates. It was clearly demonstrated that with plant maturation, the dNDF content decreased, with the last (4th) harvest (BBCH 65) showing significantly lower values compared to all other sampling times. While the crude fiber and NDF content of triticale continuously increased with advancing maturity, the proportion of digestible fiber steadily declined. In the experiment, the dNDF value measured at the first harvest (BBCH 45; 373.59 g/kg DM) decreased to 311.42 g/kg DM by the final harvest (BBCH 65).

We monitored the $NDFd_{48}$ parameter, which expresses the proportion of fiber digestible in the rumen within 48 hours, given as a percentage. This indicator reliably demonstrates how effectively the fiber fraction of triticale green biomass can be utilized in ruminant nutrition - whether it contributes valuable nutrients or primarily acts as indigestible bulk in the rumen. The highest $NDFd_{48}$ values were measured at the first harvest (BBCH 45; 67.4–73.2%), after which a continuous decline was observed with advancing plant maturity. By the fourth harvest (BBCH 65), the lowest $NDFd_{48}$ values were recorded (52.4–60.1%), representing a statistically significant decrease across all sampling dates. Among the tested varieties, significant differences in $NDFd_{48}$ were found. ’GK Maros’

consistently showed the highest fiber digestibility, while 'GK Szemes' exhibited the lowest $NDFd_{48}$ values on average across all years and harvest times, confirming its inferior digestibility performance.

Based on our results, we found that the net energy for lactation (NEI) of triticale green biomass was significantly higher during the first two harvests (BBCH 45-49). This can be attributed largely to the high proportion of digestible fiber present at these early stages. In contrast, as the crop matured and the fiber fraction became less digestible, both the NEI- and the amino acid content of the biomass declined compared to earlier harvests. For NEI, the same trend is supported by the international literature (Coblentz and Ottman, 2022). To date, no published data are available at either the national or international level regarding the amino acid composition of triticale green biomass - particularly its lysine and methionine content. Therefore, the results presented in this study represent a novel scientific contribution to the field.

Based on the evaluation of several years of experimental data, we established that with plant maturation the fiber digestibility, crude protein, NEI and amino acid content of triticale varieties decrease, while the proportion of poorly digestible fiber fractions increases, resulting in a decline in overall nutritive value.

This clearly demonstrates that substantial differences may exist among triticale varieties in terms of their forage value. Based on our comprehensive analysis, we concluded that for high-yielding dairy farms, early harvest - specifically the first cutting - is recommended for the examined Hungarian-bred triticale varieties. When considering multiple parameters together, the variety 'GK Szemes' showed the poorest forage quality traits, whereas the

varieties ’GK Maros’ and ’Dimenzio’ were characterized by more favorable quality attributes.

4. RECOMMENDATIONS FOR THE PRACTICAL USE OF THE RESULTS

We compared the androgenic response of four triticale cultivars and eight F_1 combinations using two induction media. Both media proved effective for inducing androgenesis and producing doubled haploid (DH) plants. The principal difference between the two media was their macro- and microelement composition. Further investigation and optimization of medium composition may improve the efficiency of androgenesis induction even more.

Albinism is a multifactorial phenomenon observed during *in vitro* androgenesis. It is particularly common in cereals—especially wheat and barley—under tissue culture. Numerous factors may underlie the occurrence of albino regenerants, acting alone or in interaction, including: genotype, growth conditions of donor plants, stress (cold) pretreatments, in vitro culture conditions, and molecular/genetic determinants of chloroplast development. It is also important to note that the levels of certain metal ions - such as iron, copper, and manganes - can influence the assembly and function of the photosynthetic apparatus, which is directly related to the appearance of albinism.

In the present work, albinism was addressed only tangentially; however, a dedicated future program would be warranted to elucidate this phenomenon further. To reduce albinism, it is advisable to optimize all parameters of the *in vitro* system, with particular attention to medium composition, hormonal balance, the induction protocol, and donor plant pretreatments. Although genotype is a factor that is difficult to control within a breeding program, an appropriately

selected and adapted method can still enable a high proportion of green regenerants and thus the efficient production of DH lines, which is fundamentally important from a breeding perspective.

In our experiment, we assessed the genetic homogeneity of the DH_2 generation. The majority of the DH lines proved homogeneous. Although the doubled haploid (DH) technique provides an excellent route to genetically uniform lines, 2–8% of the DH lines showed heterogeneity. We attribute this to triticale's minor but non-negligible outcrossing propensity. Accordingly, breeders should pay particular attention to strict mechanical and spatial isolation. For DH triticale lines, isolated increase and maintenance are not merely recommended but essential to ensure long-term genetic purity during variety maintenance. We wish to emphasize this critical point and recommend that breeding programs treat it as a priority. Based on our results, we recommend the triticale DH production method for routine breeding use, even in its current state of development.

Triticale as a forage crop has been a relatively under-explored area of crop production. Until recently, information on triticale green forage was scarce or very limited. Our aim was to evaluate the utilization of triticale as a forage, and to determine whether the species - and the cultivars tested - are suitable for forage production, as well as what nutritive value their forage provides.

Based on our experimental results (2017–2020), the tested triticale genotypes produced high green yields - 18.8 to 31.6 t/ha when standardized to 30% DM - therefore they can be recommended for forage production now and in the near future. It should be noted that these autumn-sown small-grain cereals, which efficiently utilize winter precipitation and have relatively low water requirements, are

unlikely to experience reductions in early-spring (April) green yield under climate change.

Based on our experimental results, we found that early harvest - BBCH 45 (young ear 4–6 cm, still enclosed in the flag-leaf sheath) - yields favorable nutritive values (crude protein, dNDF, $NDFd_{48}$, NEI, amino acids). With progressively later cuts, these values declined, while the proportion of less digestible fiber fractions increased, reducing overall nutritive value. We therefore recommend relatively early harvest (BBCH 45–49).

Given these outcomes, triticale harvested at BBCH 45–49 can be an excellent forage for high-producing dairy herds, whereas later-harvested triticale (BBCH 58–65) is more suitable for beef cattle and replacement heifers with lower nutrient demands.

Cereals - including triticale - are generally low in lysine and methionine, which can limit protein utilization. Because we found no published data (domestically or internationally) on the amino acid composition of triticale green forage, this remains a largely unexplored area, and further research is warranted.

Based on our experimental data, the triticale cultivars tested can achieve yields comparable to rye under normal production conditions when harvested in late April, and especially in early May. Finally, triticale offers a wider harvest window - it ages more slowly - so harvest can be postponed into May without compromising quality. The optimum harvest period is therefore broader than for rye, and the start of harvest can be shifted 1–2 weeks later. This also provides more favorable weather for wilting in the swath.

After triticale is removed, the field can still be re-utilized within the same season: in most years there remains sufficient time to sow early maize, sorghum, or sudangrass.

5. NEW SCIENTIFIC RESULTS

I summarize the novel findings of my research in four points:

1. **Induction media for triticale anther culture**. The P4mf and W14mf induction media are both effective for inducing androgenesis and producing doubled haploid (DH) plants in triticale cultivars and hybrids. Medium composition and genotype each had a significant effect on the numbers of green-, albino-, and transplanted regenerants, as did their interaction.

2. **Genetic homogeneity of DH lines**. Using molecular (SSR) markers, we documented the genetic homogeneity of Hungarian triticale DH lines. Owing to triticale's outcrossing propensity, 2–8% of DH lines in the DH_2 generation exhibited genetic heterogeneity. Consequently, strict mechanical- and space isolation is essential during breeding and variety maintenance in triticale.

3. **First detailed Hungarian study on forage quality dynamics**.
This is the first Hungarian study to examine, over multiple years and phenological stages, changes in the crude protein, fiber, digestible fiber, and amino acid contents of four Hungarian-bred triticale cultivars (Hungaro, Dimenzio, GK Szemes, GK Maros). Across years, with advancing plant maturity, fiber digestibility, crude protein, energy, and amino acid contents declined, while the

proportion of poorly digestible fiber fractions increased, resulting in a reduction in overall nutritive value.

4. **Harvest recommendations for feeding.**
Our integrated analysis indicates that, for high-producing dairy herds, triticale should be harvested early (at the first harvest) to maximize forage quality; later harvests are more suitable for cattle with lower nutrient demands.

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