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In vitro digestibility as screening tool for improved forage quality in triticale

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ABSTRACT

Context or Problem: Small grain forages are considered an alternative to maize, a globally important forage crop on dairy farms. Triticale is one of the most promising small grains to be cultivated as a source of forage. The inclusion of triticale in the crop rotation system can widen a narrow maize rotation, increase biodiversity, and improve the humus balance. However, successful implementation of the use of triticale forage depends largely on its forage quality, of which digestibility is the most important characteristic.
Objective or Research Question: The main objectives of this study were to investigate the genetic variation for *in vitro* digestibility (IVD) on total plant and stem level in a diverse triticale collection and to identify traits that can assist breeders in selecting for improved IVD.
Methods: The collection consisted of 120 winter triticale genotypes (103 varieties and 17 breeding lines) of North-American and European breeding origin. These genotypes were cultivated in micro plots in two consecutive growing seasons and harvested at the soft dough maturity stage (Z85, Zadoks scale). Forage quality traits were estimated on total plant and stem samples using near infrared spectroscopy (NIRS).
Results: The dataset showed considerable genotypic variation (CV 3.9–10%) and broad-sense heritability values between 0.66 and 0.73 for total plant *in vitro* digestibility of organic matter (plant IVOMD), total plant *in vitro* digestibility of neutral detergent fibre (plant IVNDFD), and stem IVNDFD. Our results show that low total plant acid detergent fibre (ADFom) can be used as a selection criterion to improve plant IVOMD and plant IVNDFD. In turn, stem Klason lignin (KL) has the highest potential for improving stem IVNDFD. Multivariate analysis combining all traits investigated, revealed clustering according to North-American or European breeding origin. In general terms, the European genotypes have higher IVD than the North-American genotypes.
Conclusions: In conclusion, high phenotypic diversity and high heritabilities were detected for triticale forage quality on total plant and stem level in a collection of European and North-American triticale.
Implications or Significance: This study suggested the potential for improvement of triticale forage IVD, indicating total plant ADFom and stem KL might be relevant traits in triticale forage breeding. Promising genotypes with a good plant IVOMD and high forage yield are of relevance for future triticale forage breeding purposes.

1. Introduction

Triticale (× *Triticosecale* Wittmack) and other small grain cereals such as wheat, oats, barley, and rye are members of the Poaceae family (Diekmann, 2009; Rutkoski et al., 2017). Primary triticale was initially developed to combine the quality characteristics of wheat, its female parent, and the robustness of rye, its pollen parent (Mergoum et al., 2019). Nonetheless, breeding efforts have mainly focussed on secondary triticale, the progeny derived from crosses between primary triticales or from crosses of primary triticale with wheat or rye (Ammar et al., 2004; Kiss, 1966). The cultivation of triticale is widespread because of its high adaptability to less fertile soils, winter hardiness, and drought tolerance

Abbreviations: aNDFom, neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash; ADFom, acid detergent fibre expressed exclusive of residual ash; BLUP, best linear unbiased predictor; CV, coefficient of variation; DM, dry matter; DMY, dry matter yield; GDD, growing degree days; H^2, broad-sense heritability; IVD, *in vitro* digestibility; IVNDFD, *in vitro* digestibility of neutral detergent fibre; IVOMD, *in vitro* digestibility of organic matter; KL, Klason lignin; MAX, maximum; MIN, minimum; NIRS, near infrared spectroscopy; OM, organic matter; PC, principal component; PCA, principal component analysis; SD, standard deviation; STA, starch.

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(Arseniuk, 2015). In 2018, triticale was cultivated on approximately 3.8 million ha worldwide, with the European countries Poland, Belarus, Germany, and France as top producers (FAOSTAT, 2023). Not unexpectedly, hardly 15% of the 442 triticale varieties listed in the Organization for Economic Co-operation and Development (OECD) database are from non-European origin, mainly from Australia, Canada, Chile, and Mexico (OECD, 2020). About 60% of the varieties listed in this database are categorised as 'winter triticale'.

Triticale is a versatile crop, mainly used as feed, but usage for food and biofuel production has also been reported (McGoverin et al., 2011). For the production of feed, triticale is grown as 'feed grain', as 'forage crop' or as 'dual-purpose crop' (Myer and Lozano del Río, 2004). Notwithstanding triticale is mainly grown as feed grain, plant breeders also have developed varieties suited for forage production which attain dry matter yields (DMY) comparable to those of other small grain cereals (Ayalew et al., 2018; Gowda et al., 2011; McGoverin et al., 2011; Salmon et al., 2004). Moreover, triticale often outyields wheat in both favourable and unfavourable growing conditions with respect to overall biomass production (Bassu et al., 2011). Its greater seedling vigour (López-Castañeda and Richards, 1994), which is associated with a more vigorous root system (Richards et al., 2007), contributes to the higher yield potential of triticale. In terms of DMY under limited water supply, triticale is one of the most promising small grains to be cultivated as source for forage. In this respect, several studies have demonstrated the higher forage production of triticale compared to wheat when grown under water-limited conditions (Estrada-Campuzano et al., 2012; Fayaz and Arzani, 2011). The greater drought tolerance of triticale can be attributed to the earliness of its heading date and to the greater capacity of its roots to withdraw water from the soil (Giunta et al., 1993).

In an agronomic context, the inclusion of cereals in the crop rotation system possesses the advantage of widening a narrow maize rotation and improving the humus balance (Haesaert et al., 2002; Księżak et al., 2018). In addition, crop rotations including winter cereals, such as winter triticale, help to prevent soil erosion and nitrate leaching into ground water because the soil is covered for most of the growing season (Braunwart et al., 2001; Haesaert et al., 2002; Ketterings et al., 2015). However, maize is still a predominant high-yielding forage crop, worldwide cultivated as source of energy in cattle diets (Ferraretto et al., 2018). Implementation of the use of cereal forages on dairy farms will depend on two main aspects: yield and quality (McCartney and Vaage, 1994). A number of region-specific studies have been conducted to determine the effect of factors influencing forage yield and quality of winter triticale (Bilgili et al., 2009; Coblentz et al., 2018; Delogu et al., 2002; Haesaert et al., 2002; Lekgari et al., 2008; Nadeau, 2007). These studies demonstrate that the forage potential of a triticale genotype is greatly influenced by the growing conditions in which it is cultivated, but also by the developmental stage at harvest.

Forage quality of small grain cereals, including triticale, is a compromise between the quantity and the digestibility of the starch component (mainly present in the ear) and the fibre component (mainly present in the stem) (Cherney and Marten, 1982a; Cherney and Marten, 1982b). Neutral and acid detergent fibre (aNDFom and ADFom respectively) in triticale forage decrease while starch (STA) increases with accumulating growing degree days (GDD) (Lyu et al., 2018). This decline is caused by a dilution effect due to increasing STA accumulation from milk to dough development of the kernels (Cherney and Marten, 1982a; Cherney and Marten, 1982b). As a result, the ear:stem ratio will increase during ripening (Khorasani et al., 1997).

A high digestibility is an important factor for the chances of success of using triticale as an alternative forage crop. The *in vitro* digestibility (IVD) of cereal forages can be accurately predicted using near infrared spectroscopy (NIRS) (Deaville et al., 2009). Given the advantage of fast, reliable, and accurate IVD estimates, NIRS can be a valuable technique for genotype screening. However, in soft dough triticale, very little is known about the variation in *in vitro* digestibility of organic matter (IVOMD) and of neutral detergent fibre (IVNDFD), exceptions represent Baron et al. (2015) and Haesaert et al. (2002). Moreover, if we want to improve triticale forage digestibility by using genetic approaches (*e.g.* predicted breeding), studying IVD at the stem level is necessary. Differences in triticale stem digestibility are expected because of genotypic variation in plant length (Baron et al., 2015; Gowda et al., 2011; Losert et al., 2017). Furthermore, in other members of the Poaceae, such as perennial ryegrass (*Lolium perenne* L.) and maize (*Zea mays* L.), the existence of such variation has been demonstrated. More specifically, genetic variation for aNDFom digestibility in perennial ryegrass and maize stems was found to be highly negatively associated with stem Klason lignin (KL) (Méchin et al., 2000; van Parijs, 2016).

The purposes of this study were (i) to investigate the genetic variation and broad-sense heritability for total plant and stem IVD in triticale forage at the soft dough maturity stage; (ii) to identify relevant subtraits that can be considered by breeders in order to select for improved IVD; (iii) to investigate whether the forage quality and yield of triticale genotypes is related to their breeding origin, and; (iv) to evaluate the forage potential of the studied triticale germplasm by considering both yield and quality. More specifically, we investigate the available variation for plant IVOMD, plant IVNDFD, and stem IVNDFD and their interrelationships in a diverse triticale collection by using micro plots at the field level. To further assist breeders in their definition of breeding targets, we also explore the links between these three parameters and the following phenomorphological, yield-related, and quality subtraits: earliness, stem length, ear length, ear proportion, DMY, STA, ADFom, and stem KL. All subtraits contribute to the characterisation of the different triticale genotypes. Data of two growing seasons are combined to get robust estimates that are less dependent of climatological conditions and breeding values are calculated as best linear unbiased predictors (BLUP values). As the suitability of a triticale variety as forage should not be just a trade-off between yield and quality, both factors need to be sufficiently high (McCartney and Vaage, 1994). Therefore, we also discuss whether it is possible to select high-yielding triticale genotypes with a high IVD.

2. Materials and methods

2.1. Plant material

The study included 120 winter triticale (× *Triticosecale* Wittmack) genotypes (Table 1), of which 103 are commercial varieties and 17 are breeding lines. Roughly 75% of the genotypes are of European origin and 25% are of North-American origin (60% from Canada and 40% from the US). The majority of the European varieties included in this collection originate from France (n = 22), Poland (n = 18), and Germany (n = 17). Two triticale hybrids (HYT Max and HYT Prime) from the HegeSaat GmbH & Co. KG breeding company (Germany) were also included. The breeding lines were developed by Field Crop Development Centre Lacombe in Canada (n = 12) and Research Farm Bottelare in Belgium (n = 5).

2.2. In-season weather conditions

The triticale genotypes were cultivated in the 2017 – 2018 and 2018 – 2019 growing seasons on experimental fields of the Ghent University's Research Farm in Bottelare, Belgium (latitude 50°57'43", longitude 3°45'37''). According to the Köppen Climate Classification system, this region has a marine west coast climate with mild differences between minimum and maximum temperatures and adequate rainfall year-round (Kottek et al., 2006). During the two seasons considered, the air temperature and rainfall were recorded by the meteorological station located at the Research Farm in Bottelare.

2.3. Field trials

Field trials were hand-sown in parcels with sandy-loam soil at a

Table 1
List of triticale varieties and breeding lines tested in Belgium during the growing seasons 2017 – 2018 and 2018 – 2019 and arranged according to their country of breeding origin.

Country of breeding origin	Breeding company	Genotype name	
Varieties			
Canada	Field Crop Development Centre Lacombe	Bobcat, Luoma, Metzger, Pika	4
Denmark	Sejet Plant Breeding	Jura, Neogen	2
France	Florimond Desprez	Kereon, Triade, Tribeca, Tricolor, Trimaran, Triskell	6
	Lemaire Deffontaines Semences	Alambic, Anagram, Bienvenu, Exagon, Oxygen	5
	RAGT Semences	Bellac, Borodine, Ragtac, RGT Eleac, RGT Expotrac, RGT Flac, RGT Omeac, RGT Ruminac, RGT Villarac, Seconzac, Tarzan	11
Germany	HegeSaat GmbH & Co. KG	Amarillo 105, Cortino, HYT Max* , HYT Prime* , Logo, Massimo, Trinidad, Vuka	8
	KWS Lochow GmbH	Cosinus, KWS Aveo, Mungis, Rhenio, Triamant, Trimmer	6
	Nordsaat Saatzucht GmbH	Claudius, SU Agendus, Tulus	3
Poland	Danko Hodowla Roslin	Atletico, Benetto, Dinaro, Fidelio, Fredro, Kasyno, Lamberto, Lasko, Madilo, Magnat, Moreno, Mundo, Orinoko, Piano, Porto, Prego, Salto, Silverado	18
Romania	INCDA-Fundulea	Cascador F, Haiduc, Negoiu, Pisc, Stil, Tulnic	6
Switzerland	Delley Seeds and Plants Ltd.	Bedretto, Blenio, Dorena, Larossa, Prader, Timbo, Trialdo, Tridel, Villars	9
The Netherlands	Lantmännen SW Seed B.V.	Adverdo, Agostino, Barolo, Cedrico, Cultivo, Dometica, Kaulos, Kortego, KWS Fido, Lombardo, Rotego, SW Talentro, Temuco	13
United States	Northern Seed Montana	10T70126, TriCal 105, TriCal 115, TriCal 131, TriCal 135, TriCal 158, TriCal 336, TriCal 348, TriCal 813, TriCal 815, TriCal Flex 719, TriCal Gainer 154	12
Breeding lines			
Belgium	Research Farm Bottelare	A12–02–430, A12–03–010, A12–03–033, A12–03–266, A12–08–290	5
Canada	Field Crop Development Centre Lacombe	00D016010, 00D016023, 02D006005, 02D006019, 03D005004, 06A049010, 06A051, 06D013002, 11B012, 11B019, 11B023, 11B025	12
* hybrids		**Total:**	120

density of 350 kernels/m^2 at a fixed depth of 2 cm. Micro plots consisted of six 1.0 m-long rows with 0.125 m inter-row spacing and were arranged according to a randomized complete block design in three replicates. Each replicate was split into two rows containing 60 genotypes (columns) each, to make the trial more compact. In November 2017, the trial was established in a field in Merelbeke, Belgium (latitude 50°57'46", longitude 3°45'35''), while in November 2018, it was laid out in a field in Oosterzele, Belgium (latitude 50°57'28", longitude 3°46'6''). Fertiliser applications followed field-specific recommendations set by the Soil Service of Belgium (https://www.bdb.be). Nitrogen was applied in three fractions at Z25, Z31, and Z39 (Zadoks scale) (Zadoks et al., 1974): in 2017 – 2018: 63 – 55 – 57 kg N/ha; in 2018 – 2019: 63 – 60 – 32 kg N/ha. Phosphorus was applied as one fraction together with potassium fertiliser at Z25 (Zadoks scale) (Zadoks et al., 1974): in 2017 – 2018: 70 kg P_2O_5/ha + 30 kg K_2O/ha; in 2018 – 2019: 89 kg P_2O_5/ha + 133 kg K_2O/ha. Herbicide, fungicide, and insecticide applications were made during the growing season following good agricultural practices. To determine the genotypic yield potential of each genotype, no plant growth regulators were used.

2.4. Data collection

Table 2 describes all traits that were determined in the triticale collection during both growing seasons.

2.4.1. Field measurements

Per micro plot, plant height was measured in cm from the ground to the tip of the ears, excluding awns, on six main culms when anthesis was completed (Torres and Pietragella, 2012). Ear length was measured in cm on the same culms with exclusion of awns. Stem length was calculated as the difference between plant height and ear length.

2.4.2. Sampling and NIRS analysis

All genotypes were harvested at the soft dough maturity stage (Z85, Zadoks scale) (Zadoks et al., 1974). This stage was determined by evaluating the development of the kernel (Pask, 2012). Due to

Table 2
Description of the traits determined in the 2017 – 2018 and 2018 – 2019 seasons in Belgium on a set of 120 triticale genotypes.

Trait (unit)	Description	Method of determination
Main traits		
Plant IVOMD (%)	Total plant *in vitro* organic matter digestibility	Determined with NIRS from De Zutter et al. (2023).
Plant IVNDFD (%)	Total plant *in vitro* neutral detergent fibre digestibility	Determined with NIRS from De Zutter et al. (2023).
Stem IVNDFD (%)	Stem *in vitro* neutral detergent fibre digestibility	Determined with NIRS from De Zutter et al. (2023).
Phenomorphological subtraits		
Plant height (cm)	Total plant length from ground level, including ear but excluding awns	Determined on 6 main culms per micro plot. Used to calculate stem length.
Ear length (cm)	Length of the ear, excluding awns	Determined on 6 main culms per micro plot. Average value considered.
Stem length (cm)	Length of the stem	Calculated as the difference between plant height and ear length on 6 main culms per micro plot. Average value considered.
Earliness (GDD)	Thermal time from mid anthesis until soft dough maturity, expressed in growing degree days	Based on field observations, and calculated for each genotype as the GDD between the moment when 50% of the plants had reached mid anthesis and the moment when 50% of the plants had reached soft dough maturity.
Yield-related subtraits		
Ear prop (%)	Percentage of ear on total plant biomass	Calculated as fraction of ears on total plant dry matter.
DMY (t/ha)	Dry matter yield	Calculated as fresh weight per micro plot multiplied by dry matter content and converted to t/ha.
Quality subtraits		
STA (g/kg DM)	Total plant starch content	Determined with NIRS from De Zutter et al. (2023).
ADFom (g/kg DM)	Total plant acid detergent fibre content, expressed exclusive of residual ash	Determined with NIRS from De Zutter et al. (2023).
Stem KL (g/kg aNDFom)	Stem Klason lignin content of the main stem part between the second and third node, expressed on stem neutral detergent fibre content	Determined with NIRS from De Zutter et al. (2023).

variability in maturity between the genotypes, the genotypes were harvested at different dates. More specifically, harvest took place from 28 June to 9 July in 2018 and from 5 July to 22 July in 2019. At harvest, the total plant biomass of the third and fourth row of the micro plots (0.25 m^2/micro plot) was hand-cut at 7 cm above ground level and weighed. First, subsamples of approximately 500 g were collected and dried in a ventilated Vötsch oven (Weiss Technik, Reiskirchen-Lindenstruth, Germany) at 65 °C to determine dry matter (DM) content at harvest. These samples were subsequently ground in a Retsch mill (Retsch Benelux, Aartselaar, Belgium), equipped with a 1 mm sieve. Total plant starch (STA), crude ash, acid detergent fibre expressed exclusive of residual ash (ADFom), *in vitro* organic matter digestibility (plant IVOMD), and *in vitro* neutral detergent fibre digestibility (plant IVNDFD) were estimated on ground samples using near infrared spectroscopy (NIRS) (De Zutter et al., 2023; the NIRS estimation accuracy is shown in Table 1, column SECV absolute). Second, subsamples of approximately 500 g were collected to determine the ear proportion on total plant dry weight. Ears were separated by hand and both fractions (ears and rest of the plant) were oven-dried to calculate the fraction of ears on the total plant dry matter. Third, from triticale plants of the second and the fifth row of each micro plot, the section between the second and the third node in the main stem was hand-cut. Stem and leaves were separated whereby stem samples consisted of the sheaths and true stem together. Stem samples were oven-dried and ground with a Fritsch cutting mill (Fritsch International, Idar-Oberstein, Germany), using a 0.5 mm sieve. These samples were then used for NIRS analysis to estimate stem IVNDFD and stem Klason lignin (KL, expressed on aNDFom basis) (De Zutter et al., 2023; the NIRS estimation accuracy is shown in Table 2, column SECV absolute).

2.4.3. Calculation of growing degree days

The temperature sum (growing degree days, GDD) was calculated, following Formula 1:

$$GDD\ (°C) = [(T_{max} + T_{min})\ /2] - T_{base} \quad (1)$$

Where T_{max} is the maximum daily air temperature, T_{min} is the minimum daily air temperature, and T_{base} is the lower threshold for development (5 °C was used as threshold temperature in this study) (Wilson and Barnett, 1983). The GDD were summed from mid anthesis (Z65, Zadoks scale) until the soft dough maturity stage (Z85, Zadoks scale) for each triticale genotype in order to assess the earliness of the genotypes (Zadoks et al., 1974). The number of GDD between the Z65 – Z85 window, rather than between sowing and Z85, was taken into account to have a clearly observable biofix as initiation point.

2.5. Data analysis

All statistical computations were performed in R software (version 3.6; R Development Core Team, 2019). Linear mixed models were built using the R package lme4 (version 1.1–31; Bates et al., 2015). To estimate best linear unbiased predictors (BLUP values) averaged over the two seasons, growing season and DM content at harvest were considered as fixed effects, while genotype was modelled as a random effect (Piepho et al., 2008). Both row and column were nested in growing season as random intercepts, following Formula 2:

$$Y = \mu + S_j + DM + G_i + SC_{jk} + SR_{jl} + e_{ijkl} \quad (2)$$

Where Y is the corrected parameter, μ is the overall mean, S_j is the effect of the j-th growing season, DM is the effect of the DM content at harvest, G_i is the effect of the i-th genotype, SC_{jk} is the interaction effect of the j-th growing season with the k-th column, SR_{jl} is the interaction effect of the j-th growing season with the l-th row, and e_{ijkl} is the residual error term.

For each trait, variance components were estimated by means of Restricted Maximum Likelihood (REML) (version 1.1–31; Bates et al., 2015). Broad-sense heritability (H^2) averaged over both growing seasons was estimated following Formula 3:

$$H^2 = \sigma^2_g / (\sigma^2_g + \sigma^2_{cs}/cs + \sigma^2_{rs}/rs + \sigma^2_e) \quad (3)$$

Where σ^2_g represents the genetic variance, σ^2_{cs} is the variance attributed to column × growing season interaction, σ^2_{rs} represents the row × growing season interaction, and σ^2_e is the residual error variance, c is the number of columns (*i.e.* 60), r is the number of rows (*i.e.* 6), and s represents the number of growing seasons (*i.e.* 2) (Holland et al., 2003). Consequently, the averaged phenotypic variance (σ^2_{ph}) among the triticale genotypes can be expressed as $\sigma^2_g + \sigma^2_{cs}/cs + \sigma^2_{rs}/rs + \sigma^2_e$.

The BLUP values were used for building regularised linear regressions (type LASSO: Least Absolute Shrinkage and Selection Operator) in order to assess relevant subtraits and their effects on the main traits, using the R package glmnet (version 2.0–5; Friedman et al., 2016). Based on variance inflation factors (VIFs), the subtrait STA was excluded due to highest multicollinearity with ADFom. In this analysis, the effect parameter represents the effect on the main trait when the subtrait is increased (in case of positive correlation) or decreased (in case of negative correlation) by 1 unit while holding all other subtraits constant. Subsequently, effect sizes were scaled by multiplying them with the standard deviation (SD) of the subtrait. The scaled value of the effect size represents the effect on the main trait when the subtrait is increased by twice the SD. In a breeding context, scaled parameters are more relevant because a highly variable subtrait has potential to improve the main trait.

To investigate whether the forage characteristics of the triticale genotypes are related to their breeding origin, a principal component analysis (PCA) was performed on the BLUP values, using the R pca3d package (version 0.10.2; Weiner, 2020). Labels for breeding origin are shown at each group's centroid. Only the ten traits with highest contribution to the total phenotypic variation were selected for representation in the PCA biplot. Finally, a scatter plot was drawn to explore whether, based on DMY and plant IVOMD, identification of promising triticale forage genotypes was possible. The mid-point for the x and y axis was calculated as the mean value of the maximum and minimum values of plant IVOMD and DMY, respectively.

3. Results

3.1. In-season weather conditions

The weather conditions during the growing seasons 2017 – 2018 and 2018 – 2019 varied throughout the different developmental stages of triticale (Supplementary Figure 1). In general, both seasons were warmer than the climatic normals in Belgium (KMI, 2021). Overall, the temperature sum in spring 2018 was remarkably high with warm April and May months (Supplementary Figure 1). In contrast, May 2019 was rather cold. The precipitation pattern was different in the seasons (Supplementary Figure 1). The summer of 2018 started exceptionally dry, and only a fraction of the climatic normal precipitation had fallen during June and July. In 2019, precipitation was more evenly distributed, but March and June were wet.

3.2. Phenotypic variation and heritability for in vitro digestbility and related traits

The summary statistics of the BLUP values, genotypic, phenotypic, and pooled error variances together with the resulting broad-sense heritability for the traits evaluated in the 2017 – 2018 and 2018 – 2019 seasons over the 120 triticale genotypes are listed in Table 3. Frequency distributions of the traits are presented in Supplementary Figure 2. Considering the main traits, plant IVD ranged from 60% to 72% for IVOMD and from 48% to 60% for IVNDFD while stem IVNDFD varied widely between 29% and 46%. Broad-sense heritability (H^2) was

Table 3
Summary statistics of BLUP values, genotypic variance, phenotypic variance, pooled error variance, and broad-sense heritability for traits evaluated over 120 triticale genotypes during the 2017 – 2018 and 2018 – 2019 seasons in Belgium. Values are the statistics for two seasons and three replicates.

Trait (unit)	Mean	SD	MIN	MAX	CV	σ^2_g	σ^2_{ph}	σ^2_e	H^2
Main traits									
Plant IVOMD (%)	67	2.6	60	72	3.9	0.07	0.11	0.04	0.66
Plant IVNDFD (%)	54	2.6	48	60	4.7	0.07	0.10	0.03	0.73
Stem IVNDFD (%)	38	**3.9**	29	46	10	0.16	0.22	0.06	0.72
Phenomorphological subtraits									
Stem length (cm)	106	17	68	154	16	296.11	311.62	15.25	0.95
Ear length (cm)	11	0.8	9	13	7.4	0.72	1.02	0.29	0.71
Earliness (GDD)	471	30	385	543	6.3	1008.16	1775.13	765.29	0.57
Yield-related subtraits									
Ear prop (%)	58	3.3	48	64	5.7	11.84	15.55	3.67	0.76
DMY (t/ha)	21	1.1	18	23	5.4	2.36	12.37	9.95	0.19
Quality subtraits									
STA (g/kg DM)	269	18	225	303	6.6	375.99	757.42	380.25	0.50
ADFom (g/kg DM)	254	14	231	293	5.4	212.23	366.99	153.92	0.58
Stem KL (g/kg aNDFom)	128	7.7	111	143	6.0	64.73	99.44	34.50	0.65

BLUP, best linear unbiased predictor; SD, standard deviation; MIN, minimum; MAX, maximum; CV, coefficient of variation; σ^2g, genotypic variance; σ^2ph, phenotypic variance; σ^2e, pooled error; H^2, broad-sense heritability; IVOMD, in vitro organic matter digestibility; IVNDFD, in vitro neutral detergent fibre digestibility; GDD, growing degree days; Ear prop, ear proportion; DMY, dry matter yield; STA, starch; DM, dry matter; ADFom, acid detergent fibre expressed exclusive of residual ash; KL, Klason lignin.

higher for plant IVNDFD than for plant IVOMD (Table 3). Moreover, H^2 was quite similar for plant and stem IVNDFD. Considering the subtraits, the coefficient of variation (CV) and H^2 were the highest for stem length, while ear length, earliness, and ear proportion varied to a lesser extent (Table 3). On average, total plant triticale forage had a higher STA than ADFom content (Table 3). Ear length, earliness, ear proportion, STA, ADFom, and stem KL showed moderate to high H^2 values that ranged between 0.50 and 0.76. Due to a high error variance compared to the genotypic variance, the heritability value for DMY was much lower (Table 3).

3.3. Effect of acid detergent fibre and Klason lignin on in vitro digestibility

Effects and scaled effects of all relevant subtraits on plant IVOMD, plant IVNDFD, and stem IVNDFD are shown in Table 4. The ADFom had the highest (scaled) effect on plant IVOMD. For example, a decrease in ADFom by 1 g/kg DM is expected to improve plant IVOMD by 0.16%. Although ear length makes a larger contribution to plant IVNDFD than ADFom (Table 4), its larger variation compensates for its lower effect. The potential genetic gain in plant IVNDFD is consequently the highest for ADFom. Lastly, stem KL clearly explained most of the variation in stem IVNDFD (Table 4). Decreasing stem KL by 1 g/kg aNDFom has the potential to improve stem IVNDFD by 0.42%.

Table 4
Regularised linear regression models (type LASSO) where plant IVOMD, plant IVNDFD, and stem IVNDFD of soft dough triticale forage are explained using averaged BLUP values: effects and scaled effects of ADFom, stem KL, stem length, and ear prop on plant IVOMD; effects and scaled effects of ADFom, stem KL, stem length, and ear length on plant IVNDFD; effects and scaled effects of ADFom, stem KL, and stem length on stem IVNDFD. Traits were evaluated over 120 triticale genotypes during the 2017 – 2018 and 2018 – 2019 seasons in Belgium.

Subtrait	Effect	2×SD[1]	Scaled effect[2]
Plant IVOMD (multiple R^2: 95.3%)			
ADFom	-0.16	27.38	-4.38
Stem KL	-0.02	15.40	-0.31
Stem length	-0.01	34.24	-0.34
Ear prop	0.03	6.66	0.20
Plant IVNDFD (multiple R^2: 89.0%)			
ADFom	-0.12	27.38	-3.29
Stem KL	-0.04	15.40	-0.62
Stem length	-0.03	34.24	-1.03
Ear length	0.16	1.64	0.26
Stem IVNDFD (multiple R^2: 92.8%)			
ADFom	-0.02	27.38	-0.55
Stem KL	-0.42	15.40	-6.47
Stem length	-0.02	34.27	-0.68

SD, standard deviation; IVOMD, *in vitro* organic matter digestibility; IVNDFD, *in vitro* neutral detergent fibre digestibility; ADFom, acid detergent fibre expressed exclusive of residual ash; KL, Klason lignin; Ear prop, ear proportion
[1] twice the standard deviation
[2] effect multiplied with 2×SD

3.4. Influence of breeding origin on triticale forage potential

To get an overview of the relationships between the studied traits, a PCA was performed using the BLUP values. Because of poor contribution to the total phenotypic variation, ear length could be removed from the dataset without loss of information (Supplementary Figure 4). Almost 78% of the total phenotypic variance was explained by the first two PCA components (Fig. 1). Separation of the genotypes along PC 1 was determined in particular by plant IVOMD, plant IVNDFD, ear proportion, and stem IVNDFD, which were positively correlated among each other, but negatively correlated to the other discriminant parameters ADFom, stem length, and stem KL (Fig. 1). Among the traits that contribute to separation along PC 2, earliness was negatively correlated to DMY and STA, as indicated by the opposite direction of the arrows (Fig. 1). The biplot clearly showed lack of correlation between DMY and the IVD traits. Furthermore, the ellipses illustrate that triticale genotypes of similar geographical origin tend to group together (Fig. 1). Genotypes originating from North-America (The United States and Canada) showed high ADFom content. Genotypes of Central European origin (Romania, Poland, and Switzerland) are late varieties with a high stem IVNDFD. Northwest European genotypes (Denmark, France, Germany, and Belgium) had high STA content and high DMY. Genotypes from The Netherlands showed high plant and stem IVNDFD, and a high ear proportion.

3.5. Improving forage potential by selecting high-yielding triticale genotypes with a high in vitro digestibility

Plant IVOMD was plotted against DMY in order to identify high-yielding genotypes with a high IVD (Fig. 2). In this graph, genotypes were colour-coded based on ADFom content, which was identified as the main subtrait determining plant IVOMD (Table 4). In general, the triticale genotypes were distributed over the four quadrants (Fig. 2). The BLUP values of the triticale varieties and breeding lines for plant IVOMD, DMY, and ADFom are presented in Supplementary Table 1.

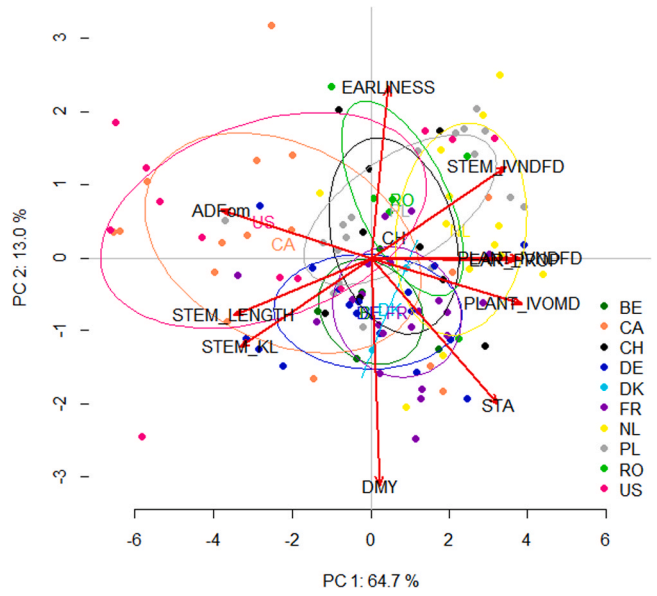

Fig. 1. Visualisation of the results from the principal component analysis for traits of triticale genotypes grown in the 2017 – 2018 and 2018 – 2019 seasons in Belgium, using averaged BLUP values. The biplot showed genotype separation along the first two principal components, PC 1 (64.7%) and PC 2 (13.0%). Genotypes are colour-coded based on their country of breeding origin: BE, Belgium; CA, Canada; CH, Switzerland; DE, Germany; DK, Denmark; FR, France; NL, the Netherlands; PL, Poland; RO, Romania; US, United States. Labels of breeding origin are shown at each group's centroid. Trait names are shown on top of the loading vectors: DMY, dry matter yield; STA, starch; plant IVOMD, total plant *in vitro* organic matter digestibility; plant IVNDFD, total plant *in vitro* neutral detergent fibre digestibility; stem IVNDFD, stem *in vitro* neutral detergent fibre digestibility; Earliness; ADFom, acid detergent fibre; Ear prop, ear proportion; stem KL; stem Klason lignin; stem length.

Superior genotypes with DMY > 22 t/ha (Q3-value, 3rd quartile) and plant IVOMD > 69% (Q3-value) were A12–02–430 (BE), Kaulos (NL), Kereon (FR), KWS Fido (NL), Rhenio (DE), and Trialdo (CH) (Fig. 2). Temuco (NL) had the highest plant IVOMD and lowest ADFom content, but DMY was below 22 t/ha. Nevertheless, the other triticale genotypes in quadrant I are interesting breeding material too (DMY > 20.46 t/ha and plant IVOMD > 65.89%) (Fig. 2). The genotypes in quadrant II (n = 22) have high yield potential but poor IVOMD (DMY > 20.46 t/ha and plant IVOMD < 65.89%) (Fig. 2). Overall, the hybrid HYT Max (DE) had the highest DMY (Supplementary Table 1). Triticale genotypes in quadrant IV (n = 18) showed good IVOMD but have a DMY below 20.46 t/ha (Fig. 2). Genotypes in quadrant III (n = 13) are less useful for forage purposes (DMY < 20.46 t/ha and plant IVOMD < 65.89%) (Fig. 2). The Canadian varieties Bobcat and Metzger were characterised by the lowest DMY and lowest plant IVOMD respectively (Supplementary Table 1).

4. Discussion

4.1. Phenotypic variation and heritability for in vitro digestbility and related traits

The warm and dry weather conditions during the first year of field trials (growing season 2017 – 2018) seemed to accelerate the triticale development, the genotypes flowered earlier than in the experiment carried out in the growing season 2018 – 2019 (Supplementary Figure 1). In addition, high temperatures and water shortage reduce the grain filling period in triticale (Estrada-Campuzano et al., 2008; Santiveri et al., 2002). As a consequence of the early flowering and probably also of drought and heat stress during grain filling, harvest in 2018 started much sooner than in 2019 (Supplementary Figure 1). During two growing seasons, field evaluation of triticale genotypes of different breeding pools revealed considerable variation for total plant and stem IVD (Table 3, Supplementary Figure 2). This illustrates a significant influence of triticale genotype on IVD at the soft dough maturity stage (De Zutter et al., 2023; Haesaert et al., 2002). Previous studies similarly revealed the presence of substantial variation for total plant IVD in perennial ryegrass varieties and in maize lines (Beecher et al., 2015; Méchin et al., 2000). However, cereal crops consist of ears and stems together. For breeding purposes, it is therefore important to distinguish between the influences of a higher proportion of ears (compared to the proportion of stems) by weight and an intrinsic higher digestibility of the stem fraction (stem IVNDFD), as these are two alternative/complementary breeding approaches. We have demonstrated the presence of substantial differences for stem IVNDFD in triticale (Table 3), similarly to what was demonstrated by Méchin et al. (2000) in maize and by van Parijs (2016) in perennial ryegrass. Due to their high heritability (H^2, Table 3), the IVD traits are considered mostly

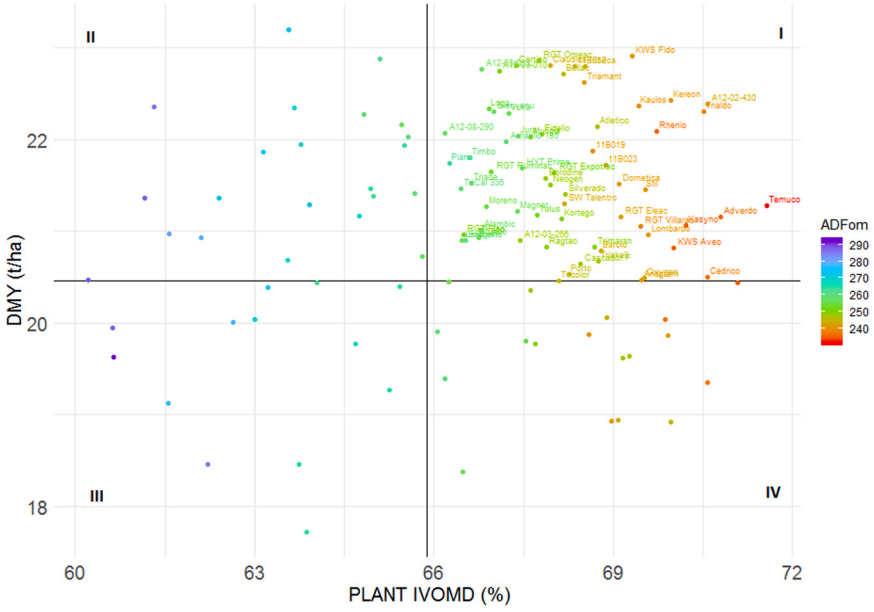

Fig. 2. Scatter plot showing genotype separation along plant IVOMD (*in vitro* organic matter digestibility, %) and DMY (dry matter yield, t/ha), evaluated over 120 triticale genotypes in the 2017 – 2018 and 2018 – 2019 seasons in Belgium. Genotypes are colour-coded based on ADFom (acid detergent fibre expressed exclusive of residual ash, g/kg DM) content. The BLUP values for plant IVOMD, DMY, and ADFom averaged over the two seasons were used. The mid-point for the x and y axis is calculated as the mean value of the maximum and minimum values of plant IVOMD and DMY, respectively. The most promising triticale forage genotypes (DMY > 20.46 t/ha and plant IVOMD > 65.89%) are labelled. I, II, III, IV: quadrant number.

genetically controlled. However, it should be mentioned that the high H^2 values are partially due to the narrow environmental matrix in this study. To further understand the genetic basis of IVD in this collection, a genome wide association approach will be employed in a follow-up study.

4.2. Effect of acid detergent fibre and Klason lignin on in vitro digestibility

The high H^2 values for the IVD traits also reflect their potential for improvement in the studied triticale germplasm. Our results revealed ADFom as the most relevant subtrait for improving plant IVD (Table 4), due to its high negative correlation with plant IVOMD and with plant IVNDFD (Spearman correlation matrix is given in Supplementary Figure 3). De Boever et al. (1999) demonstrated the same link between IVOMD and ADFom in maize silage. To improve stem IVNDFD, it is more adequate to focus on stem-specific subtraits. We determined KL in the triticale stem since it is a measure for the total lignin content of the plant cell wall. Moreover, stem KL was found to offer the highest breeding potential for improving stem IVNDFD (Table 4). This is not surprising as stem digestibility is generally determined by the digestibility of the cell wall components, on which the lignin fraction has a strong negative influence (Jung and Allen, 1995; Jung et al., 2012). Indeed, the high negative effect of stem KL on stem IVNDFD (Table 4) proved that lignin content is the major factor making the cell wall undigestible (Barrière et al., 2003). Digestibility of aNDFom in maize and perennial ryegrass stem fractions was also shown to be highly negatively correlated with KL (Méchin et al., 2000; van Parijs, 2016).

It can be stated that decreasing total plant ADFom or stem KL in forages will cause an increase in digestibility which means that ruminants are more able to utilize the nutrients that are present in the forage (Eskandari et al., 2009; Lithourgidis et al., 2006). Indeed, Baron et al. (2015) suggested that triticale forage quality may be improved by increasing the starch-rich ear proportion or by decreasing the lignin-rich stem proportion. This can be achieved by elevating the crop cutting height at harvest (Walsh et al., 2008). By reducing the stem fraction and thus increasing the ear fraction, aNDFom and ADFom content were consequently reported to be significantly decreased in barley forage (Kim et al., 2016). On the other hand, past breeding efforts have been successful in reducing plant length in triticale (Losert et al., 2017; Oettler, 2005). Nonetheless, the use of shorter triticale genotypes may reduce forage yield (Baron et al., 2015). Therefore selection of genotypes low in ADFom, which is already practiced in Canadian triticale forage breeding programmes, would be a more valuable option to improve plant IVD (Aljarrah et al., 2014; Baron et al., 2015). A way to improve stem IVNDFD is by breeding for triticale genotypes with lower lignin content in the stem. A lack of lignin could lead to reduced agronomic fitness such as lodging and hence a balance between a better stem digestibility and a lower lodging susceptibility should be taken into account (Jung et al., 2012; Wang et al., 2014).

4.3. Influence of breeding origin on triticale forage potential

Our study included a diverse set of 120 triticale genotypes, mostly of important European breeding origin (Table 1). Although other triticale breeding origins such as Hungary were not represented, the collection is of relevance for European winter triticale (Ammar et al., 2004). Furthermore, genotypes originating from breeding pools in Canada and the United States were also included (Table 1). In the PCA biplot (Fig. 1), classification of the triticale genotypes according to their country of breeding origin revealed grouping by North-American and European origin. This illustrates that breeders have developed breeding material specifically adapted to their region, have similar breeding objectives and/or made use of the same genetic material to establish triticale breeding programmes. For example, the triticale breeding programme at the Field Crop Development Centre in Canada focuses on the development of high-yielding forage lines with improved quality characteristics. Despite low ADFom and high cell wall digestibility were used as selection criteria (Aljarrah et al., 2019; Baron et al., 2015), we found that Canadian and American genotypes (Table 1) were associated with a rather high ADFom content (Fig. 1). In consequence, the North-American triticale genotypes had lower IVD than genotypes from European breeding pools. Northwest European genotypes have high DMY potential (Fig. 1). Supporting this finding, in a triticale collection mainly containing German elite germplasm, large variation for DMY at the early dough stage and potential for improvement of this trait was shown (Gowda et al., 2011). However, the Dutch genotypes (Table 1), showing good IVNDFD characteristics, tended to group separately (Fig. 1).

4.4. Improving forage potential by selecting high-yielding triticale genotypes with a high in vitro digestibility

There is a lack of knowledge about the selection and breeding of triticale genotypes that combine good forage yield and quality. In this study, the variation in DMY and IVD was investigated in a representative collection of winter triticale since winter types have higher forage yield than spring types due to their longer growth cycle and their higher tillering rate (Mergoum et al., 2009; Myer and Lozano del Río, 2004; Royo, 1997). The DMY did not show any significant correlation with IVD (PCA biplot in Fig. 1 and Spearman correlation matrix in Supplementary Figure 3), which is consistent with the results obtained by Aljarrah et al. (2014) and Haesaert et al. (2002). Several varieties and breeding lines tested in the present study combined high DMY with a good IVOMD (Supplementary Table 1) and might have great potential to be used as source for triticale forage breeding programmes. Their good forage potential is related to their low ADFom content (scatter plot in Fig. 2). Several varieties that were identified as superior were actually not bred for forage purposes (Mergoum and Gómez-Macpherson, 2004). It is nonetheless remarkable that only one American variety, TriCal 336, was identified as a promising genotype (Supplementary Table 1). Even though those varieties were typically bred for forage use, they may not be well adapted to Northwest European growing conditions because some exhibited extreme stem lengths (> 125 cm) (Supplementary Figure 2) (https://tricalforage.com/). On the other hand, improved IVD has been crossed in Canadian breeding lines (Aljarrah et al., 2019) and revealed the promising lines 11B012, 11B019, and 11B023 (Supplementary Table 1). In contrast, Bobcat, a Canadian triticale variety released about 20 years ago, had low performance as forage crop (Supplementary Table 1) which is in close agreement with the findings of Baron et al. (2015). Recent breeding efforts have aimed to develop triticale hybrids that have higher grain yield and plant height as a result of heterosis (Longin et al., 2012). One of the two hybrids in our study, HYT Max (DE), certainly excels as a forage producer (Supplementary Table 1).

4.5. Agronomic implications

These field studies, conducted under temperate climate conditions, provided valuable information on the forage performance of a diverse set of triticale genotypes. Despite the experiments were carried out in a limited geographic region (Belgium, Northwest Europe), our results our potentially applicable to similar global environments. Since our investigations were conducted over only two growing seasons, during which the temperatures were quite warm, more field studies are be needed to verify the potential of triticale on the long term. In addition, other weather data such as precipitation and radiation can be added in future analyses to help understand how weather conditions could influence triticale forage yield and quality. We have demonstrated variability for both forage yield and forage quality, with genotype selection found to be an important agronomic factor. It seems possible to increase triticale forage IVD by selecting for a lower ADFom content of the total plant or for a lower KL content of the stem. However, agronomic

performance may be reduced with a lower stem KL content due to increased lodging susceptibility. Nevertheless, further exploration is necessary to confirm this hypothesis. Furthermore, the findings reported in this manuscript demonstrate the potential of on-farm application of triticale forage, but further evaluations are needed for its validation. Further research can investigate the effect of crop cultivation techniques that can influence the IVD of triticale forage, such as sowing density and fertilisation, without compromising forage yield.

5. Conclusions

To breed new triticale varieties that have superior forage quality, the availability of genotypic variation for this trait is essential. Our dataset showed considerable variation and a high heritability for total plant *in vitro* digestibility of European and North-American triticale genotypes. Triticale forage *in vitro* digestibility is, however, a complex trait. Hence, the effects of several subtraits related to the ear and stem were investigated. Interestingly, acid detergent fibre was shown to mainly explain total plant *in vitro* digestibility of neutral detergent fibre, and of organic matter and can consequently be used as selection criterion. Similarly as for other forages, Klason lignin was found a breeding target for improved *in vitro* digestibility of the triticale stem. To be successfully implemented on dairy farms, triticale forage genotypes should have both high yield and high *in vitro* digestibility. In this respect, our field experiments pinpointed promising triticale forage genotypes, which opens up new forage breeding avenues.

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CRediT authorship contribution statement

Anneleen De Zutter: Conceptualization, Methodology, Data curation, Formal analysis, Investigation, Writing – original draft, Visualization, Project administration, Johan De Boever: Conceptualization, Writing – review & editing, Supervision, Hilde Muylle: Conceptualization, Methodology, Visualization, Writing – review & editing, Resources, Supervision, Isabel Roldán-Ruiz: Conceptualization, Methodology, Visualization, Writing – review & editing, Supervision, Geert Haesaert: Conceptualization, Methodology, Visualization, Resources, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fcr.2023.109009.

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