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Prospects for tritordeum (×*Tritordeum martinii* A. Pujadas, Nothosp. Nov.) cereal breeding: Key points for future challenges

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Abstract

Tritordeum is the new cereal derived from crosses between the wild barley *Hordeum chilense* Roem. et Schultz. and either durum or bread wheat, resulting in hexaploid and octoploid tritordeums, respectively. The success of tritordeum as a crop depends on the effectiveness of its breeding programme. In this work, new advanced tritordeum lines are screened for grain carotenoid content and disease susceptibility to analyse the impact of the current breeding strategies and to identify their strengths and putative limitations for future challenges. We conclude that selection for grain carotenoid content, the main strength for the tritordeum commercialization, should be reinforced not only by using the diversity of *H. chilense* but also incorporating beneficial alleles from durum wheat. Furthermore, genes for stem rust resistance from the A and B wheat genomes must be incorporated into tritordeum breeding programme. Finally, when selecting for threshability, tritordeums without chromosome substitutions should be preferentially selected using a marker-assisted selection approach.

KEYWORDS
carotenoids, food colour, threshability, tritordeum breeding

1 | INTRODUCTION

The term tritordeum (×*Tritordeum martinii* A. Pujadas, Nothosp. Nov.) refers to the amphiploids derived from crosses between the wild barley *Hordeum chilense* Roem. et Schultz. as seed parent and either durum or bread wheat as pollen donors (Martin & Chapman, 1977; Martin & Sanchez-Mongelaguna, 1982). Although both hexaploid and octoploid tritordeum genotypes were used in tritordeum breeding programmes, only hexaploid genotypes are currently cultivated because they showed lower frequency of aneuploids, higher fertility and they outperformed octoploid tritordeums for agronomic performance (Martin et al., 1996, 1999). Tritordeum showed promising agronomic and quality traits as detailed in recent reviews (Ávila et al., 2021; Landolfi & Blandino, 2023).

The main limitation for the cultivation of this new species was the non-free threshability inherited from its wild parent. To overcome this

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limitation, an inter-specific crossing scheme between hexaploid tritordeum and common wheat was used to eliminate the undesirable *H. chilense* genes two decades ago. This resulted in the selection of the first free-threshing tritordeums, characterized by the presence of the chromosome substitutions 2D/(2H^{ch}) or 5D/(5H^{ch}) (Atienza, Martín, & Martín, 2007). This advancement paved up the way for the development of tritordeum as a new crop. Subsequently, free-threshing tritordeums with the full chromosome set from *H. chilense* (hereinafter referred as complete tritordeums, AABBH^{ch}H^{ch}) were obtained. This allowed the registration of the first complete tritordeums 'Aucan' and 'Bulel', in the European Community Plant Variety Office (CPVO) which are commercially available by Vivagram B.V. (http://www.vivagram.nl). It is currently unclear whether chromosome substitutions for free threshability are still playing a role in our tritordeum breeding programme or if they have been fully overcome by complete tritordeums with free threshability. Clarifying this point is crucial for the future registration of new tritordeum varieties.

The tritordeum varieties that have been registered so far ('Aucan', 'Bulel' and 'Coique') are considered suitable for organic farming due to their resistance/tolerance to biotic stresses. Hexaploid tritordeums have shown immune response against septoria tritici blotch (STB) caused by *Zymoseptoria tritici* (Martin et al., 1996). This immunity was independent of the plant stage, as evidenced by artificial inoculations at both seedling and adult plant stages (Martin et al., 1996). Chromosome 4H^{ch} harbours the main genes responsible for this resistance (Rubiales et al., 2000). The good level of resistance conferred by this chromosome led to the development of wheat–*H. chilense* chromosome substitution lines (Calderón et al., 2012). At present, tritordeums have shown low susceptibility to this pathogen, as evidenced in field trials in the Czech Republic from 2008 to 2012 (Martinek et al., 2013). Hexaploid tritordeum typically performs well in Córdoba (Spain) against several rust. However, due to the rapid evolution of plant pathogens and the climate change, it is important to constantly monitor disease resistance (Singh et al., 2023). In this context, it would be of interest to determine the degree of resistance in locations with different race spectrum, as well as against other diseases such as stem rust (SR) which typically occur too late in Córdoba to cause significant problems.

Finally, the nutritional characteristics of tritordeum have been crucial for the successful commercialization of this new crop (Ávila et al., 2021; Landolfi & Blandino, 2023). According to Shewry et al. (2023), several quality parameters of tritordeum fall within the range of durum wheat and common wheat diversity. However, tritordeum has lower gluten content and ω-gliadins than bread wheat, making it an attractive option for general consumers whiling to reduce their gluten intake, although it should be noted that tritordeum is not suitable for those with celiac disease (Vaquero et al., 2018). Additionally, the golden colour of tritordeum-derived products is a distinguishing characteristic of this new crop and is highly appreciated by consumers. Accordingly, this is a major trait contributing to the commercialization of tritordeum (https://www.tritordeum.com) as 'the Golden cereal'. Carotenoids, mainly lutein, are responsible for the yellow pigment content of tritordeum grains (Atienza, Ballesteros, et al., 2007). Primary tritordeums are those directly obtained from the chromosome doubling of the hybrid between *H. chilense* and durum wheat as described by (Martin & Sanchez-Mongelaguna, 1982). According to Atienza, Ballesteros, et al. (2007), primary tritordeums have, on average, over 5.5 times the grain carotenoid content of their durum wheat parents. Considering the huge differences between primary tritordeums and their durum wheat parents, and that the golden colour is easily visualized in tritordeum flours, no selection for carotenoid content has been performed despite this is the most distinctive trait for its commercialization. In fact, breeding efforts have been mainly directed towards the improvement of threshability and yield. Therefore, it is important to investigate whether the lack of selection for carotenoid content in the last decade has produced undesired effects on this trait. Thus, the aim of this study was to expand the panel of tritordeum genotypes by screening new lines and focusing on grain carotenoid content while addressing potential vulnerabilities against selected biotic stresses.

2 | MATERIALS AND METHODS

2.1 | Plant materials, DNA isolation and genotyping

Twenty advanced lines derived from the tritordeum breeding programme conducted by Prof. A. Martín at IAS-CSIC (Córdoba, Spain) (HT511, HT514, HT515, HT517, HT518, HT519, HT520, HTC10.16, HTC14.16, HTC25.16, HTC26.11, HTC28.15, HTC30.13, HTC34.14, HTC36.17, HTC40.17, HTC48.15, HTC55.17, HTC38.17 and HTC58.16) along with four previously developed tritordeums (HT427, HT479, HT490 and HT507) were selected for genotypic and phenotypic characterization. All of them are free threshing. The origin of these materials is shown in Data S1.

Genomic DNA was extracted using the CTAB method (Murray & Thompson, 1980) following the detailed procedure described in (Rodríguez-Suárez et al., 2022). The molecular markers used to detect the D and H^{ch} chromosomes were selected as described by Atienza, Martín, and Martín (2007). D genome-specific chromosome markers (WMS 337-1D, WMS 261-2D, WMS 52-3D, WMS 174-5D and WMS 44-7D) developed by Röder et al. (1998) and Gdm127-6D (Pestsova et al., 2000) were used. *H. chilense*-specific chromosome markers were also used (k01779-1H^{ch}, k01360-2H^{ch}, k01242-4H^{ch}, k01323-5H^{ch}, k01062-6H^{ch} and Xat-7H^{ch}) (Hagras et al., 2005; Nasuda et al., 2005; Requena-Ramírez et al., 2020) and Gpw7553-3H^{ch} (Sourdille, 2004). PCR amplifications were performed according to the manufacturer's instructions using MyTaq™ DNA polymerase (Bioline, London, UK). Amplification conditions were as described by Atienza, Martín, and Martín (2007). PCR amplification products were resolved in 2% agarose gels stained with Safeview™ Nucleic Acid Stain (NBS biologicals, Ltd., Cambridgeshire, England) using 1× TAE buffer. Gel images were acquired with Bio-Rad Molecular Imager VersaDoc MP 4000 System equipment and analysed with Quantity One software (Bio-Rad Laboratories, Inc., USA).

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FIGURE 1 Location of the experimental sites (created with Datawrapper; © OpenStreetMap contributors).

2.2 | Field trials

During the first season (2020–2021), the advanced lines and the previously developed tritordeums were evaluated for yellow rust (YR) and SR resistance under natural infestations at Foggia (Italy) following a completely randomized block design with three replications and 1 m rows with 20–25 seeds. Disease severity (DS) was assessed using a 0–9 scale, with 0 representing no symptoms and 9 representing 100% disease score. In addition, all genotypes were multiplied at Fiorenzuola d'Arda in Northern Italy and in Córdoba, in Southern Spain, for further studies in the next season. Figure 1 shows the location of the experimental sites.

During the second year (season 2021–2022), eight advanced lines (HT514, HT515, HT517, HT518, HT520, HTC26.11, HTC38.17 and HTC58.16) were studied at Fiorenzuola d'Arda (Italy) and Santaella (Spain) for the evaluation of carotenoid content and profile. These lines were selected according to their performance in the previous season in the multiplication trial conducted in Fiorenzuola d'Arda.

The field trial at Fiorenzuola d'Arda (Italy) followed a randomized block design with three replications and 3.6 m^2 plots. In this trial, STB symptoms were visualized, and therefore, the genotypes were evaluated using the double-digit scale (Eyal & Levy, 1987).

At Santaella, a completely randomized block design with two replications with 3.6 m^2 plots was used, including 'Aucan' as control. The general agronomic practices of each region were followed at each location. After harvesting, grains were mechanically threshed and used for carotenoid content and profile determination at IG-CSIC (Seville, Spain).

2.3 | Carotenoid analysis

The protocol for extraction and analysis of carotenoids has been previously described (Mínguez-Mosquera & Hornero-Méndez, 1993; Rodríguez-Suárez et al., 2022). Briefly, 1 g of grain sample was ground with 6 mL of acetone containing 0.1% (w/v) butylated hydroxytoluene (BHT) in a stainless steel grinding jar together with two stainless steel balls (15 mm Ø) using an oscillating ball mill Retsch model MM400 (Retsch, Haan, Germany) at 25 Hz for 1 min.

The resulting slurry was transferred to a 15 mL polypropylene centrifuge tube, and the samples were centrifuged at 4500 × *g* for 5 min at 4°C. Subsequently, the supernatant was then transferred to a 15 mL tube and evaporated using a gentle stream of nitrogen. The dry extract was dissolved in 0.5 mL of high-performance liquid chromatography (HPLC) grade acetone and centrifuged, prior to chromatographic analysis, in a 1.5 mL microcentrifuge tube at 13,000 × *g* for 5 min at 4°C. Samples were extracted in duplicate. All operations were performed under dimmed light to avoid isomerization and photodegradation of carotenoids.

Carotenoid analysis was performed using an HPLC system consisting of a Waters e2695 Alliance chromatograph equipped with a Waters 2998 photodiode array detector, and controlled by Empower2 software (Waters Cromatografía, S.A., Barcelona, Spain). A C18

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reversed-phase analytical column (Mediterranea SEA18, 3 μm, 20 × 0.46 cm; Teknokroma, Barcelona, Spain) was used. Separation was achieved by binary-gradient elution with an initial composition of 75% acetone and 25% deionized water. The gradient profile used was linearly increased to 95% acetone in 10 min, held for 7 min, then increased to 100% in 3 min, and held constant for 13 min. Initial conditions were reached in 5 min. An injection volume of 10 μL and a flow rate of 1 mL/min were used. Detection was performed at 450 nm, and the online spectra were acquired in the 350–650 nm wavelength range. Quantification was performed using calibration curves generated with pigment standards. Calibration curves were generated in the concentration range of 0.5–45.0 μg/mL and were constructed by plotting peak area versus pigment concentration. Lutein esters were evaluated as free lutein equivalents. Similarly, the concentration of (*Z*)-isomers of lutein was determined using the calibration curve for (all-*E*)-lutein. Analyses were performed in duplicate and on the same day as the extracts were prepared. Data were expressed as μg/g fresh weight (μg/g fw).

Analyses of variance were performed using Statistix version 10.0 (Analytical Software, Tallahassee, FL, USA). Differences for total grain carotenoid content were determined using Tukey's honestly significant difference (HSD) test at ($p < .05$) after analysis of variance using Statistix v. 9.0. Disease resistance graphs were generated using the ggplot2 package in RStudio 2023.12.1 Build 402.

3 | RESULTS

3.1 | Prevalence of chromosome substitution lines for threshability in tritordeum breeding programme

A set of 20 advanced lines were selected at IAS-CSIC considering their good threshability. After the genotypic characterization and the study of the presence/absence of the chromosomes H^{ch} and D, only seven lines were found to be complete tritordeums (Table 1). The remaining lines presented 2D/($2H^{ch}$) chromosome substitution (eight lines), 5D/($5H^{ch}$) substitution (four lines) or both substitutions simultaneously (one line; HT515).

3.2 | Disease assessment

Disease assessments for YR and SR in Foggia during the 2020–2021 season are shown in Figure 2 and in Data S2. The line HT518 was discarded for further evaluation due to its poor germination. While tritordeum lines showed acceptable levels of resistance to YR (disease susceptibility ≤2), with the exception of the breeding lines HTC26.11 and HT519 which were highly susceptible, most tritordeum lines were highly susceptible to SR (Figure 2). Only three lines, HT514, HTC28.15 and HTC38.17, showed low DS, and thus, they can be

TABLE 1 Genotypic characterization for D and H^{ch} chromosomes of tritordeum genotypes.

	Presence of H^{ch}/D chromosomes in each homoeologous group (1 to 7)							
Genotype	1	2	3	4	5	6	7	Type[a]
HTC10.16	$1H^{ch}$	$2H^{ch}$	$3H^{ch}$	$4H^{ch}$	$5H^{ch}$	$6H^{ch}$	$7H^{ch}$	Complete
HTC14.16	$1H^{ch}$	$2H^{ch}$	$3H^{ch}$	$4H^{ch}$	$5H^{ch}$	$6H^{ch}$	$7H^{ch}$	Complete
HTC25.16	$1H^{ch}$	$2H^{ch}$	$3H^{ch}$	$4H^{ch}$	$5H^{ch}$	$6H^{ch}$	$7H^{ch}$	Complete
HTC28.15	$1H^{ch}$	2D	$3H^{ch}$	$4H^{ch}$	$5H^{ch}$	$6H^{ch}$	$7H^{ch}$	S. subs.
HTC30.13	$1H^{ch}$	$2H^{ch}$	$3H^{ch}$	$4H^{ch}$	5D	$6H^{ch}$	$7H^{ch}$	S. subs.
HTC34.14	$1H^{ch}$	2D	$3H^{ch}$	$4H^{ch}$	$5H^{ch}$	$6H^{ch}$	$7H^{ch}$	S. subs.
HTC36.17	$1H^{ch}$	$2H^{ch}$	$3H^{ch}$	$4H^{ch}$	5D	$6H^{ch}$	$7H^{ch}$	S. subs.
HTC40.17	$1H^{ch}$	$2H^{ch}$	$3H^{ch}$	$4H^{ch}$	$5H^{ch}$	$6H^{ch}$	$7H^{ch}$	Complete
HTC48.15	$1H^{ch}$	2D	$3H^{ch}$	$4H^{ch}$	$5H^{ch}$	$6H^{ch}$	$7H^{ch}$	S. subs.
HTC55.17	$1H^{ch}$	2D	$3H^{ch}$	$4H^{ch}$	$5H^{ch}$	$6H^{ch}$	$7H^{ch}$	S. subs.
HT511	$1H^{ch}$	$2H^{ch}$	$3H^{ch}$	$4H^{ch}$	5D	$6H^{ch}$	$7H^{ch}$	S. subs.
HT514	$1H^{ch}$	2D	$3H^{ch}$	$4H^{ch}$	$5H^{ch}$	$6H^{ch}$	$7H^{ch}$	S. subs.
HT515	$1H^{ch}$	2D	$3H^{ch}$	$4H^{ch}$	5D	$6H^{ch}$	$7H^{ch}$	D. subs.
HT517	$1H^{ch}$	2D	$3H^{ch}$	$4H^{ch}$	$5H^{ch}$	$6H^{ch}$	$7H^{ch}$	S. subs.
HT518	$1H^{ch}$	$2H^{ch}$	$3H^{ch}$	$4H^{ch}$	5D	$6H^{ch}$	$7H^{ch}$	S. subs.
HT519	$1H^{ch}$	$2H^{ch}$	$3H^{ch}$	$4H^{ch}$	$5H^{ch}$	$6H^{ch}$	$7H^{ch}$	Complete
HT520	$1H^{ch}$	$2H^{ch}$	$3H^{ch}$	$4H^{ch}$	$5H^{ch}$	$6H^{ch}$	$7H^{ch}$	Complete
HTC26.11	$1H^{ch}$	$2H^{ch}$	$3H^{ch}$	$4H^{ch}$	$5H^{ch}$	$6H^{ch}$	$7H^{ch}$	Complete
HTC38.17	$1H^{ch}$	2D	$3H^{ch}$	$4H^{ch}$	$5H^{ch}$	$6H^{ch}$	$7H^{ch}$	S. subs.
HTC58.16	$1H^{ch}$	2D	$3H^{ch}$	$4H^{ch}$	$5H^{ch}$	$6H^{ch}$	$7H^{ch}$	S. subs.
Aucan	$1H^{ch}$	$2H^{ch}$	$3H^{ch}$	$4H^{ch}$	$5H^{ch}$	$6H^{ch}$	$7H^{ch}$	Complete

[a]S. subs., single chromosome substitution; D. subs., double chromosome substitution; complete, tritordeums with the full chromosome set from *Hordeum chilense*.

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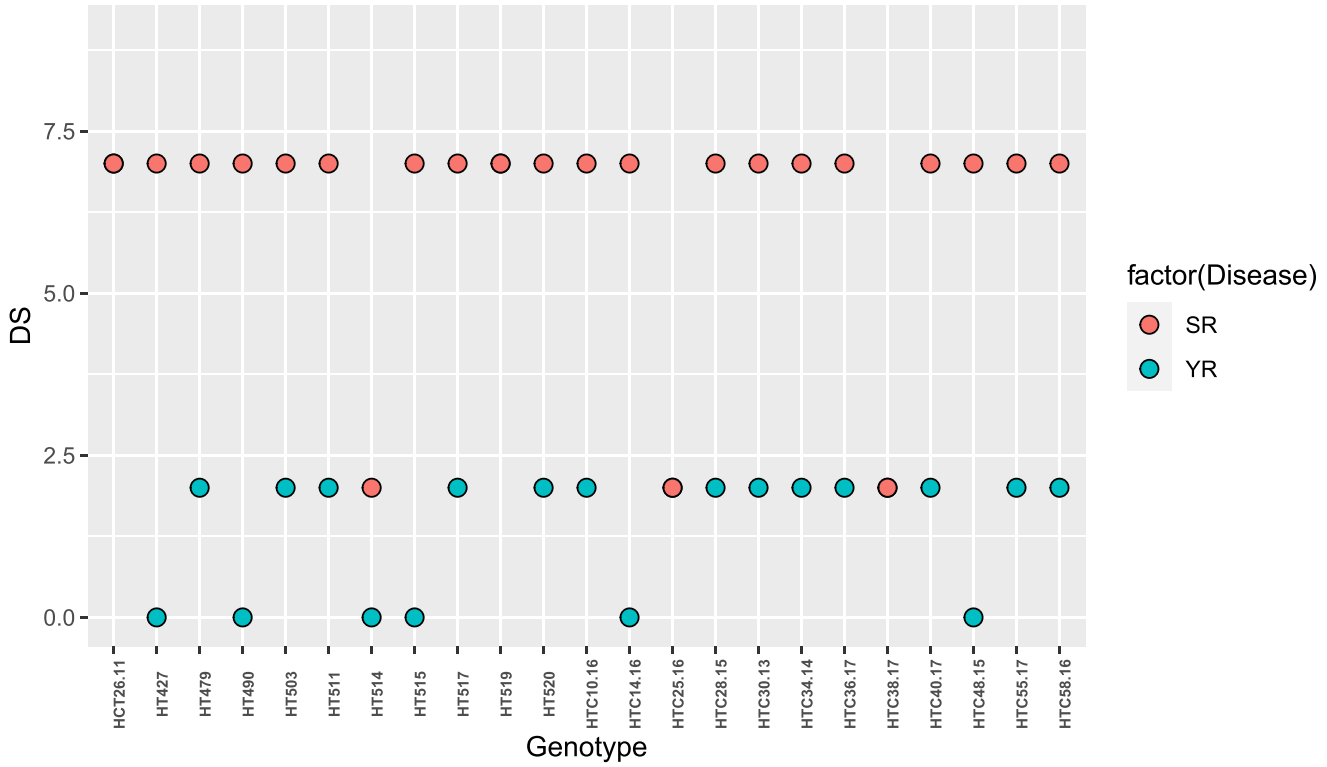

FIGURE 2 Disease assessment for yellow rust (YR) and stem rust (SR) at Foggia (Italy) during the season 2020–2021. For each genotype and disease, the maximum disease score considering the three replicates is shown. Disease severity (DS) was assessed using a 0–9 scale, where 0 stands for no symptoms and 9 for total area affected by the disease.

considered as resistant. Nevertheless, these results should be confirmed in future experiments to discard the possibility of lack of infestation, because disease incidence depended on the natural occurrence of the pathogen and not on field inoculations.

During the 2020–2021 season, advanced tritordeum lines were propagated at CREA-Fiorenzuola d'Arda (Italy) and at Santaella (Spain). Eight lines (HT514, HT515, HT517, HT518, HT520, HTC26.11, HTC38.17 and HTC58.16) were selected for further experimentation based on their agronomic behaviour (data not shown). The field trial at Fiorenzuola d'Arda showed symptoms against STB, and therefore, all the lines were evaluated (Figure 3 and Data S3). Most lines showed acceptable disease susceptibility scores (disease susceptibility below 3.0 and blotch development restricted to bottom leaves). However, HT518 was highly susceptible in all three replicates with STB just below the flag leaves (Figure 3).

3.3 | Grain carotenoid content

The carotenoid profile and content of grains was determined in the 2021–2022 season in the advanced tritordeum lines studied at CREA-Fiorenzuola d'Arda (Italy) and at Santaella (Spain) (Table 2). The detailed carotenoid profile included lutein as the major pigment, either free or esterified with fatty acids, accounting between 93.9% and 96.6% of total carotenoids (Data S4). Minor amounts of zeaxanthin and β-carotene were also found.

The average carotenoid content of grains cultivated at Santaella (Spain) was higher than at Fiorenzuola d'Arda (Italy), although the interaction Genotype × Environment was not significant ($p = .9179$), and thus, the ranking of grain carotenoid content was similar in both locations (Figure 4). Grain carotenoid content at Santaella varied between 5.13 (HT517) and 8.77 μg/g (HT514). Although HT514 and HTC58.16 did not differ from 'Aucan' at $p = .05$, most of the lines were clearly below 'Aucan' for grain carotenoid content.

4 | DISCUSSION

The first tritordeum lines to overcome the threshability problem were developed by inter-specific crosses between common wheat and tritordeum (Atienza, Martín, & Martín, 2007). Those genotypes carried chromosomal substitutions (either 2D/(2H^{ch}) or 5D/(5H^{ch}) or even both at the same time), and their good threshability was supposed to be related to the absence of the wild homoeologues of the *Tenaceous glumes* (*Tg*) and the domestication factor (Q) genes (Atienza, Martín, & Martín, 2007) on Chromosome Groups 2 and 5, respectively. It is important to emphasize that both 'Aucan' and 'Bulel', which were registered at the CPVO office in 2017 and 2015, respectively, are complete tritordeums. Thus, our hypothesis was that, at present,

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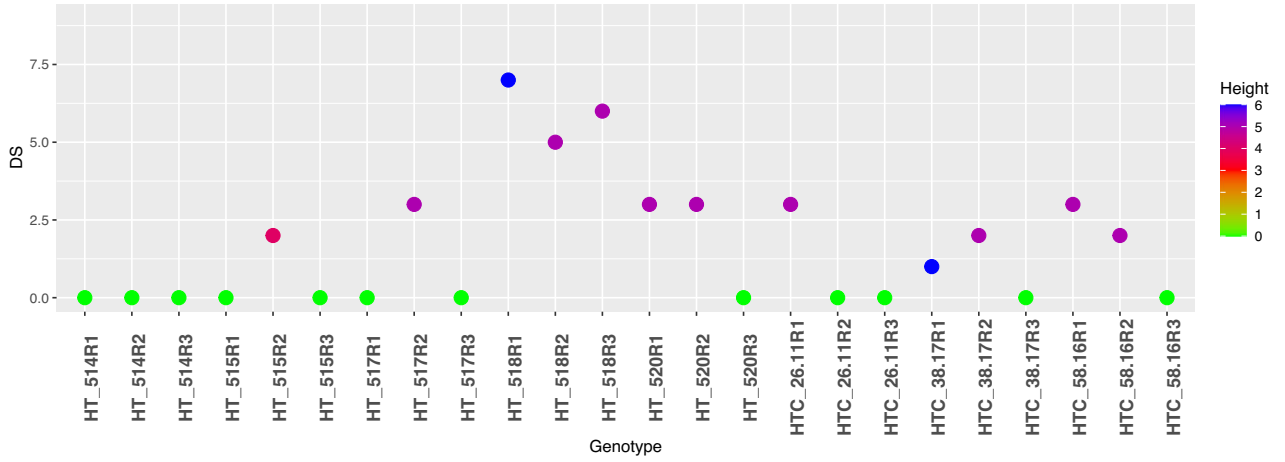

FIGURE 3 Assessment of septoria tritici blotch (STB) incidence in selected advanced tritordeum lines at Fiorenzuola d'Arda (Italy) during the 2021–2022 season. Disease severity (DS) was assessed using a 0–9 scale, where 0 stands for no symptoms and 9 for total area affected by the disease. A secondary scale was used (0–9), indicating blotch development in terms of plant height, where 0–3 stands for bottom leaves, 5 if the disease reached the middle (50%) of the plant height, 8 for reaching the flag leaf and 9 for reaching the spike.

TABLE 2 Grain carotenoid content (μg/g fw) of selected tritordeum advanced lines during 2021–2022 season at Fiorenzuola d'Arda (Italy) and Santaella (Spain).

Genotype	Location	FLut	RCar	LutME	LutDE	TCar
HT514	Fior	4.438 ± 0.13^{ab}	0.213 ± 0.01^{a}	1.072 ± 0.11^{a}	0.175 ± 0.03^{ab}	5.898 ± 0.25^{a}
HT515	Fior	3.222 ± 0.52^{bc}	0.170 ± 0.01^{abc}	0.727 ± 0.27^{a}	0.155 ± 0.09^{ab}	4.274 ± 0.88^{ab}
HT517	Fior	2.464 ± 0.12^{cd}	0.153 ± 0.01^{bc}	0.489 ± 0.05^{a}	0.090 ± 0.01^{ab}	3.196 ± 0.18^{b}
HT518	Fior	1.835 ± 0.12^{d}	0.133 ± 0.01^{c}	0.719 ± 0.05^{a}	0.325 ± 0.02^{a}	3.013 ± 0.17^{b}
HT520	Fior	3.724 ± 0.36^{abc}	0.198 ± 0.02^{ab}	0.828 ± 0.05^{a}	0.212 ± 0.02^{ab}	4.963 ± 0.40^{ab}
HTC26.11	Fior	3.737 ± 0.35^{abc}	0.169 ± 0.01^{abc}	0.906 ± 0.20^{a}	0.198 ± 0.06^{ab}	5.010 ± 0.59^{ab}
HTC38.17	Fior	3.627 ± 0.13^{abc}	0.207 ± 0.00^{a}	0.540 ± 0.03^{a}	0.073 ± 0.01^{b}	4.448 ± 0.14^{ab}
HTC58.16	Fior	4.603 ± 0.20^{a}	0.205 ± 0.01^{ab}	1.081 ± 0.06^{a}	0.124 ± 0.02^{ab}	6.014 ± 0.23^{a}
Aucan	Sant	8.430 ± 0.49^{a}	0.434 ± 0.05^{a}	0.573 ± 0.04^{d}	0.012 ± 0.00^{e}	9.449 ± 0.58^{a}
HT514	Sant	7.122 ± 0.12^{b}	0.442 ± 0.00^{a}	1.141 ± 0.09^{a}	0.066 ± 0.01^{bc}	8.771 ± 0.03^{ab}
HT515	Sant	5.259 ± 0.03^{bc}	0.352 ± 0.01^{abc}	0.700 ± 0.04^{bcd}	0.039 ± 0.01^{cde}	6.350 ± 0.08^{de}
HT517	Sant	4.135 ± 0.10^{bc}	0.312 ± 0.01^{bc}	0.648 ± 0.01^{cd}	0.038 ± 0.00^{cde}	5.134 ± 0.11^{e}
HT518	Sant	4.058 ± 0.01^{c}	0.263 ± 0.00^{c}	1.063 ± 0.05^{a}	0.195 ± 0.00^{cde}	5.579 ± 0.04^{e}
HT520	Sant	6.221 ± 0.07^{c}	0.392 ± 0.01^{ab}	0.938 ± 0.01^{ab}	0.091 ± 0.00^{b}	7.641 ± 0.06^{bcd}
HTC26.11	Sant	5.814 ± 0.21^{cd}	0.417 ± 0.01^{ab}	0.906 ± 0.04^{abc}	0.0036 ± 0.00^{de}	7.173 ± 0.25^{cd}
HTC38.17	Sant	5.495 ± 0.32^{d}	0.0366 ± 0.02^{abc}	0.614 ± 0.07^{d}	0.037 ± 0.01^{cde}	6.511 ± 0.42^{de}
HTC58.16	Sant	6.541 ± 0.25^{d}	0.385 ± 0.02^{ab}	1.132 ± 0.01^{a}	0.057 ± 0.00^{cd}	8.115 ± 0.26^{abc}

Note: Mean values ± SE. For each location and pigment, different letters indicate significant differences ($p < .05$) determined by Tukey's HSD test. The detailed carotenoid compositions are available in Data S4.

Abbreviations: Fior, Fiorenzuola d'Arda (Italy); FLut, free lutein = (*all-E*)-lutein + (*Z*)-lutein isomers; LutDE, lutein diesters = lutein dilinoleate + lutein linoleate-palmitate + lutein dipalmitate; LutME, lutein monoesters = lutein monolinoleate + lutein monopalmitate; RCar, rest of carotenoids = (*all-E*)-zeaxanthin + (*all-E*)-α-carotene + (*all-E*)-β-carotene; Sant, Santaella (Spain); TCar, total carotenoids.

chromosome substitutions related to free threshing would currently play a minor role in the selection for free threshability. However, this assumption proved to be wrong, as 63% of the advanced lines selected for free threshing were chromosome substitution lines. The good threshability of these lines was explained by the interaction between *Tg* and *Q* loci (Kerber & Rowland, 1974). The *Q* gene pleiotropically affects other genes such as the *Tg* locus in wheat (Simons et al., 2006). The cloning and characterization of the *Q* gene in wheat (Simons et al., 2006) showed that this gene belongs to the APETALA2 (AP2) family of transcription factors. Further studies in *H. chilense* and

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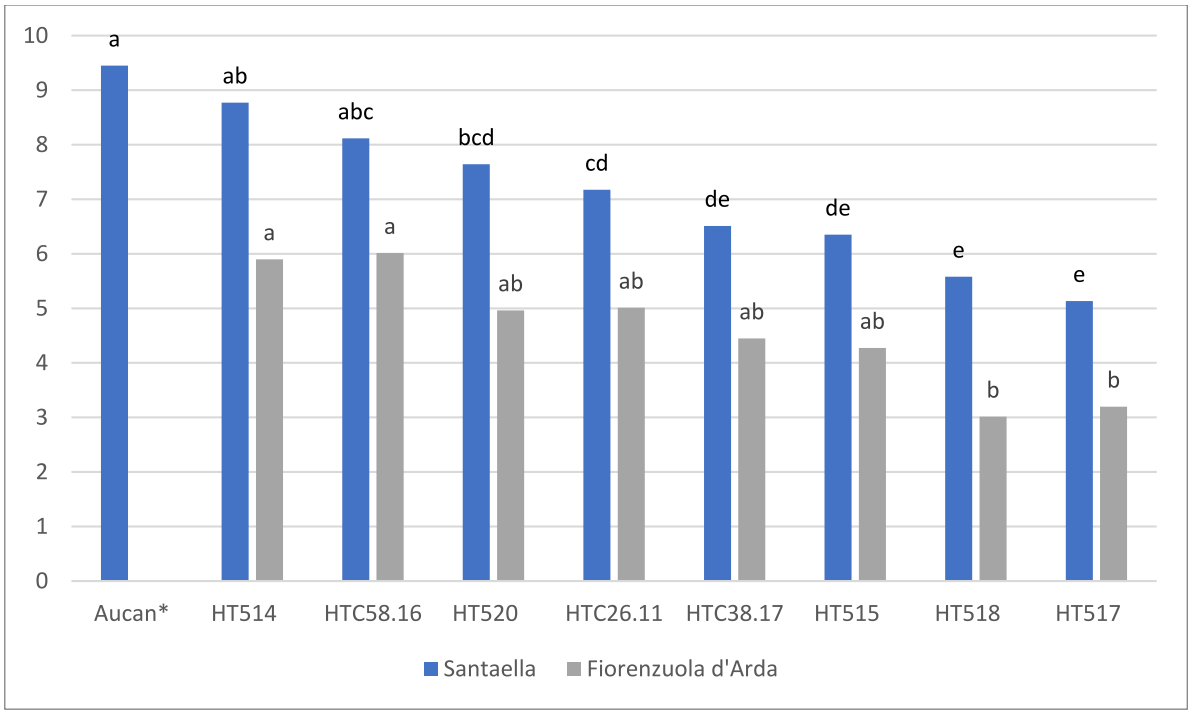

FIGURE 4 Total grain carotenoid content (μg/g fw) at Fiorenzuola d'Arda (Italy) and Santaella (Spain) during the 2021–2022 season. For each location, different letters indicate significant differences ($p < .05$) determined by Tukey's HSD test. *Aucan was not available for Fiorenzuola d'Arda field trials.

tritordeum allowed the cloning and characterization of an *AP2*-like gene similar to the Q wheat gene in chromosome $5H^{ch}$ (Gil-Humanes et al., 2009). As expected, the chromosome substitution line $5D/(5H^{ch})$, developed by Atienza, Martín, and Martín (2007), did not show expression of the *AP2*-like gene because chromosome $5H^{ch}$ was absent in this line. Besides, the $2D/(2H^{ch})$ chromosome substitution also showed very low levels of *AP2*-like gene expression during spike development, suggesting that the absence of chromosome $2H^{ch}$ exerts a pleiotropic effect on the *AP2*-like gene (Gil-Humanes et al., 2009). The high prevalence of chromosome substitution lines should be corrected using a marker-assisted selection approach coupled with selection for free threshability, because the special characteristics of tritordeum are conferred by the H^{ch} genome.

Tritordeum varieties are recommended for organic farming in their commercial brochures, as they are tolerant or resistant to a wide range of biotic stresses (Martin et al., 1996). However, the results shown here for SR are very concerning, as this disease is considered to be one of the major threats to wheat production at present. The high susceptibility of tritordeum advanced lines against SR clearly indicates the need to incorporate new resistance genes in the breeding programme. It is important to note that tritordeum behaves like wheat with regard to rust diseases (Martin et al., 1996). Therefore, resistance sources should be transferred from either durum or common wheat. The identification of the isolate Ug99 (now known as TTKSK race) in Uganda in 1998 as virulent to the widely deployed *Sr71* along with many other *Sr* genes (Preorius et al., 2000), encouraged the search for new sources of resistance to this pathogen. According to the Global Rust Reference Center (https://wheatrust.org), the TTRTF race was predominant in Italy during the 2020–2021 season. Although this race was detected in Spain in the 2020–2023 period, our results demonstrate the urgent need for breeding efforts in tritordeum to include resistance sources against this race. Hexaploid tritordeums carry both A and B genomes. Therefore, resistance genes from these genomes can be easily transferred to tritordeum. Breeding for resistance requires the combination of genes with broader and more durable resistance, such as the slow-rusting adult plant resistance gene *Sr17* (Zhang et al., 2017). According to the latest report for SR from the Global Rust Reference Center, this gene is effective against the TTRTF race (Hovmoller et al., 2023). Besides, it has been cloned (Zhang et al., 2017), which would facilitate the development of a marker-assisted strategy using already established protocols (https://maswheat.ucdavis.edu/protocols/Sr17). However, other sources of resistance located on the A and B genomes should also be considered, as there are currently 12 recognized SR genetic groups with different virulence phenotypes (Hovmoller et al., 2023).

Regarding STB, the high susceptibility of HT518 should be seriously considered in tritordeum breeding, although other factors may have influenced the susceptibility of this particular line. Previously, it had been shown that hexaploid tritordeums showed an immune response against STB independently at both seedling and adult plant stages (Martin et al., 1996). This resistance is conferred by genes located in chromosome $4H^{ch}$ (Rubiales et al., 2000) and the good performance against STB has been a constant during the last two decades. In fact, 10 tritordeums showed high resistance against STB

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during a 5-year assessment (2008–2012) in the Czech Republic (Martinek et al., 2013). The high susceptibility of HT518 at Fiorenzuola d'Arda makes it important to remain vigilant against this pathogen in tritordeum breeding.

The detailed carotenoid profile (available in Data S4) is consistent with previous studies in tritordeum and wheat (Atienza, Ballesteros, et al., 2007; Ávila et al., 2021; Colasuonno et al., 2019; Landolfi & Blandino, 2023). The golden colour of tritordeum-derived products is of paramount importance for their commercialization. Thus, the declining in grain carotenoid content observed in the breeding lines should be corrected. This is particularly important considering the existence of experimental wheat lines with high lutein content such as DM5685*B12 (Ahmad et al., 2013), with 6.3 μg/g lutein content (Ahmad et al., 2015), and yellow pigmented varieties such as 'Citrus' and 'Bona Vita' with 1.85 and 5.6 μg/g grain carotenoid content, respectively (Paznocht et al., 2019; Sardella et al., 2023), or the existence of carotenoid biofortification programmes in common wheat (Requena-Ramírez et al., 2023; Rodríguez-Suárez et al., 2022). Carotenoid-biofortified common wheat could then compete with tritordeum for the wheat colour market. Thus, further selection efforts in tritordeum breeding programme should not rely exclusively on the H^{ch} genome, but they should incorporate alleles for high grain carotenoid content available in durum wheat, at least for the *Psy1 (Phytoene synthase 1)* gene which is the main responsible for carotenoid content in Triticeae species (Atienza, Avila, & Martín, 2007; Colasuonno et al., 2019; Ficco et al., 2014). These efforts are essential for the commercialization of tritordeum because golden colour, determined by grain carotenoid content, is the main attribute for the distinction of its derived food products.

5 | CONCLUSIONS

The tritordeum breeding programme should strengthen the strategies for improving grain carotenoid content, the most distinctive trait of this species, by exploiting not only the diversity of the H^{ch} genome but also by incorporating beneficial alleles available in durum wheat. Besides, it should develop effective strategies to incorporate resistance sources against SR from the A and B wheat genomes to be ready to respond to the arrival of new pathogenic races; finally, it should refine the breeding procedures for threshability to favour the selection of complete tritordeums over chromosome substitution lines.

AUTHOR CONTRIBUTIONS

Cristina Rodríguez-Suárez: Investigation; writing—review and editing. **María Dolores Requena-Ramírez:** Investigation; writing—review and editing. **Elisabetta Mazzucotelli:** Methodology; investigation; writing—review and editing. **Anna Maria Mastrangelo:** Methodology; investigation; writing—review and editing. **Ilaria Marcotuli:** Investigation; writing—review and editing. **Agata Gadaleta:** Funding acquisition; investigation; writing—review and editing. **Antonio Martín:** Resources; investigation; writing—review and editing. **Dámaso Hornero-Méndez:** Methodology; investigation; writing—review and editing. **Sergio G. Atienza:** Conceptualization; methodology; writing—original draft; funding acquisition; investigation; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the Supporting Information of this article.

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