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Effects of hormone concentrations on anther cultures and the acquisition of regenerated plants of five awnless triticale genotypes

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Abstract

Background The rapid production of doubled haploids by anther culture technology is an important breeding method for awnless triticale. The aim of this study was to explore the effects of triticale genotype and the types and ratios of exogenous hormones in the medium on the efficiency of triticale anther culture.

Results Anthers of five triticale genotypes were cultured on four different callus induction media and the calli were induced to differentiate into green plants by culture on three different differentiation media. The triticale genotype T8004 showed the best performance in anther culture, with a callus induction rate of 28.64%, a green plantlet differentiation frequency of 33.33%, and a green plantlet production rate of 2.78%. The highest callus induction rates were obtained by culturing anthers on C3 medium (the main components were potassium nitrate, glutamine, inositol, etc.), and the highest green plantlet differentiation frequency was obtained by culturing calli on D2 differentiation medium (the main components were potassium nitrate, ammonium nitrate, calcium chloride dihydrate, etc.). Flow cytometry analyses showed that 15 of the 20 DH0 generation plants that grew normally in the field were doubled haploids. The average chromosome doubling success rate was 55.6%. Analyses of agronomic traits showed that the 11 DH1 doubled haploid plants reached the standard for awnless triticale, so they are candidate materials for breeding new awnless triticale varieties.

Conclusion The anther culture technology of triticale was optimized in this paper, which made it possible to rapidly breed homozygous varieties of awnless triticale.

Keywords Awnless triticale, Anther culture, Genotypes, Hormone concentrations, Ploidy identification, Agronomic trait

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Background

As a high-quality annual forage grass, triticale (×*Triticosecale* Wittmack) plays important roles in alleviating the conflict between forage and livestock in pastoral areas, protecting the ecological environment, and promoting the healthy development of grassland agricultural ecosystems [1, 2]. At present, most triticale varieties (lines) have awns — bristle-like appendages located on the spikelet. Although awns have roles in increasing the photosynthetic area, improving transpiration, and pest resistance [3, 4], they also have some disadvantages. The waxy awn of triticale can puncture the oral cavity of livestock, which can lead to diseases such as pharyngitis, oral ulcers, and submandibular edema [5, 6]. When triticale is harvested at the dough stage, the waxy awn can adversely affect the production of high-quality forage products and reduce the palatability of forage [7]. Awnless triticale does not have these shortcomings. Moreover, many studies have shown that the seed yield of awnless varieties is not lower than that of awned varieties [8, 9]. Therefore, it is of great significance to breed awnless triticale lines to improve the palatability of forage and the quality of forage products.

In many breeding methods, anther culture is an indispensable rapid breeding technology. It has become a powerful tool for rapid haploid production [10, 11]. Anther culture technology involves in vitro culture of microspore-containing anthers from a heterozygous generation. The microspores switch from the gametophyte development pathway to the sporophyte development pathway and form callus. Haploid plants develop from the callus, and are then subjected to a chromosome-doubling treatment, giving rise to double haploid plants. Varieties with excellent traits are selected as hybrid parents, and homozygous plants are obtained from F1 plants with improved agronomic traits by anther culture followed by chromosome doubling. This doubled-haploid breeding strategy is more efficient than traditional breeding methods [12]. Homozygotes of triticale with relatively stable traits can be obtained quickly by anther culture. This can save a lot of breeding time and reduce the labor and financial resources required for breeding [13, 14]. At present, the anther culture technique is the most widely used method for haploid breeding of triticale. In recent years, researchers have used anther culture technology to produce haploid and doubled haploid plants of various species, most commonly wheat (*Triticum aestivum*) [15], rice (*Oryza sativa*) [16], corn (*Zea mays*) [17], and oat (*Avena sativa*) [18]. Few studies have focused on the production of doubled haploid triticale. In the few studies that have used triticale in anther culture, the experimental materials were all awned triticale varieties (lines), rather than awnless ones. In the present study, all the experimental materials were awnless triticale lines. The overall goal was to provide a reference for the optimization of anther culture technology for awnless triticale.

Among the many factors that affect the efficiency of triticale anther culture, plant genotype is the most important one [19]. The success rate of anther culture varies greatly among different varieties (genotypes) because of the dependence of anther culture on genotype [20]. Marciniak et al. [21] reported that the number of green plants induced to form in anther culture differed significantly among 38 winter triticale genotypes (range, 1.0–16.2/spike) under the same culture conditions. Similarly, there were significant differences in the number of haploid green plants obtained after anther culture of 21 Swedish and Dutch F1 and F2 triticale combinations under the same culture conditions. The average number of haploid green plants ranged from 0.8 to 13.6/spike, and there were significant differences in the number of green plants induced among the lines [22]. The results of those studies indicate that the triticale genotype significantly affects the efficiency and success rate of anther culture. The type, ratio, and concentrations of exogenous hormones also affect the efficiency of anther culture [23], and the optimum types, concentrations, and ratios of hormones for anther culture differ among different plants. Kruppa et al. [24] found that P4mf medium was more suitable than W14mf medium for the production of doubled haploid triticale lines, and the addition of 1.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/L kinetin (KT) to P4mf medium significantly increased the number of calli and green plants (103.7/anther and 19.7/anther, respectively). Żur et al. [25] reported that the addition of 0.5 mg/L naphthaleneacetic acid (NAA) and 0.5 mg/L KT to C17 medium significantly increased the callus induction rate of eight triticale varieties, and 'DH144' triticale had the highest regeneration rate of green plants (5.4 green seedlings differentiated per 100 calli). Hassawi et al. [26] tested the effects of seven auxins and four cytokinins in triticale anther culture, and found that the anthers showed the highest callus differentiation rate (13.9%) on medium supplemented with 2,4-D. Katarzynaapachota [27] found that the combination of 2.0 mg/L 2,4-D and 0.5 mg/L KT in the medium significant improved the callus regeneration rate of triticale anthers. Therefore, optimization of hormone concentrations in the medium can improve the success rate in anther culture. We hypothesized that modifying the medium hormone concentration may alter the awnless triticale anther callus induction rate and green plantlet differentiation frequency, which can affect the production of haploid plants. Haploid plants are transformed into double haploid (DH) plants by spontaneous chromosomal doubling, or chromosome doubling with colchicine to obtain double haploid plants, thereby accelerating the breeding process (Fig. 1). In this study, we analyzed the effects of different types and ratios

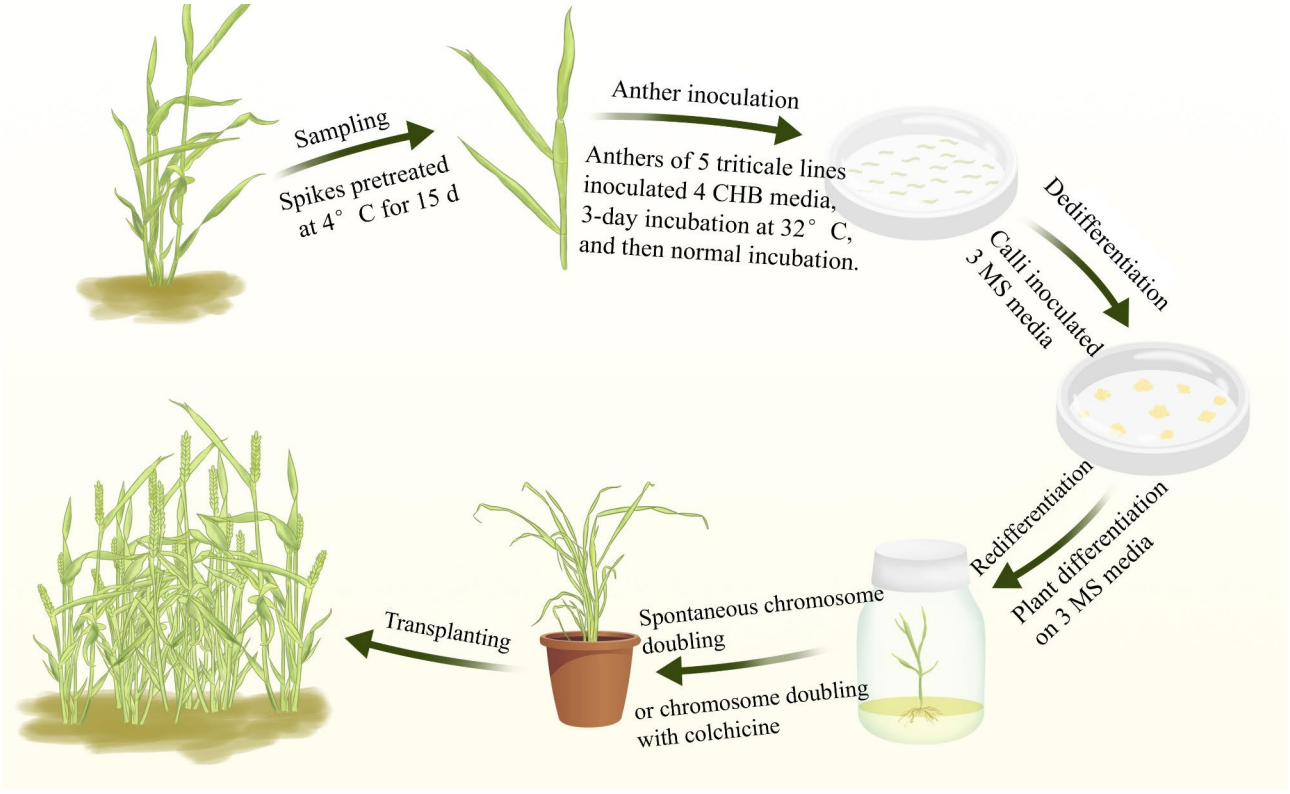

Fig. 1 Schematic of awnless triticale anther culture

Table 1 Basic information of the experimental site

Experimental site	Mean annual temperature (℃)	Frostless period (d)	Mean annual rainfall (mm)	Soil organic matter (mg/kg)	Rapidly available nitrogen (mg/kg)	Rapidly available phosphorus (mg/kg)	Rapidly available potassium (mg/kg)	pH	Soil type
Forage test station	7.9	171	349.9	2.3	90.05	7.36	172.8	7.35	loessal soil

of hormones on the callus induction rate and plant differentiation rate of five different triticale lines in anther culture. The results provide a theoretical basis for improving anther culture and optimizing the regeneration of awnless triticale.

Materials and methods

Site description

This study was conducted at the Forage Experimental Station of Gansu Agricultural University (36°03′N, 103°53′E, 1560 m above sea level), Lanzhou, China in 2021. The previous crop was high-yield sweet sorghum (Sorghum bicolor) grown under good irrigation conditions. Basic information on the experimental site is shown in Table 1.

Experimental materials

The five materials used in this experiment were the new hybrid lines of awnless triticale selected by pedigree method in Gansu Agricultural University, and the line number, name and parents were shown in Table 2. After a 30-day vernalization, the seeds of 5 awnless triticale lines were planted in the plastic greenhouse experimental base of Gansu Agricultural University (36°03′N, 103°53′E; 1,560 m above sea level), China, in March 2021. A line seeding method was adopted, with row spacing of 20 cm, plant spacing of 5 cm, and a sowing depth of 2 ~ 3 cm. 300 kg·hm^{-2} of diammonium phosphate was applied before sowing, and 196 kg·hm^{-2} of urea were applied at the regreening stage and jointing stage, respectively. During the experiment, weeding was carried out, and

Table 2 Basic information of 5 awnless triticale materials

Number	Name	Parents (♀ × ♂)
T8001	T2021-8001	OH2276 (2013) × J9 (2013)
T8002	T2021-8002	81S37 (2013) × J35 (2013)
T8003	T2021-8003	4009 (2013) × P2 (2013)
T8004	T2021-8004	T17 (2013) × J18 (2013)
T8005	T2021-8005	89D-8 (2013) × J10 (2013)

irrigation was given once at the seedling stage, tillering stage and jointing stage. Sampling began in late May and early June.

Sampling and pretreatment of young spikes

The young spikes of awnless triticale with simply microspores in the middle and late stages of mononuclear were taken (i.e., spikes of awnless triticale in the field just reached the middle of the flag leaf and the second leaf from the top). The triticale tillers were cut 10 cm below the second leaf from the top. Bundles of tillers were wrapped with aluminum foil, and the collection time and triticale line name were marked. Two anthers from the collected per young spikes were crushed on a glass slide, after which 1 ~ 2 drops of acetic acid magenta dye (Solarbio Inc., Beijing, China) were added and the stained material was examined using the Panthera U microscope (eyepiece 10× and objective 100×; Motic China Group Co., Ltd., Hong Kong, China) to determine whether uninucleate stage had been reached. The incision of the young spikes which was up to the standards by microscopic examination was inserted into a 500 mL beaker filled with 250 mL of tap water, and pretreated at 4 °C for 15 days in the dark.

Spike disinfection and anther inoculation

After 15 days of low temperature pretreatment, the flag leaf and the second leaf from the top were cut off. In the biological safety cabinet, disinfected the surface of the test material with 75% alcohol for 1 min, peeled off the young spikes, disinfected with 2% sodium hypochlorite solution for 7 min, rinsed with sterile water for 4 ~ 5 times, and then wiped the surface water of the young spikes with sterile filter paper. Anthers were removed from the sterilized spikes and placed on each of the 4 different CHB induction medium (denoted as C1, C2, C3, C4, respectively). Every 40 anthers were inoculated into a dish (90 mm diameter) (Jingan Inc., Shanghai, China), and each 6 ~ 8 dishes per tested induction medium.

Based on the hormone content of the optimal CHB induction medium screened by our team in the previous study, the hormone content was further adjusted and refined, and 4 kinds of induction medium were designed, each of which was supplemented with 90 g/L sucrose and 7 g/L agar, respectively, with a pH value of 5.4. The hormone content of 4 induction media was shown in Table 3.

Induction culture of anther

After the inoculation was completed, the petri dish was placed in a incubator (HGZ-250, Yuejin Inc., Shanghai, China) for a 3-day incubation at 32 °C in darkness, and then transferred to another incubator for a 60-day incubation at 28 °C in darkness. After 60 days, the number of callus was counted.

Table 3 Hormone concentration of induction medium

Number	Hormone concentration
C1	1.5 mg/L 2,4-D + 1.0 mg/L KT
C2	1.5 mg/L 2,4-D + 1.5 mg/L KT
C3	2.0 mg/L 2,4-D + 1.5 mg/L KT
C4	2.5 mg/L 2,4-D + 1.5 mg/L KT

Table 4 Hormone concentration of differentiation medium

Number	Hormone concentration
D1	1.0 mg/L IAA + 1.0 mg/L 6-BA
D2	1.0 mg/L IAA + 1.5 mg/L 6-BA
D3	1.0 mg/L IAA + 2.0 mg/L 6-BA

Regeneration medium

Based on the hormone content of the optimal MS differentiation medium screened by our team in the previous study, 3 differentiation medium were designed by adjusting different hormone contents. The hormone content of the 3 differentiation medium was shown in Table 4. The MS medium formulation was MS medium 4.74 g/L+sorbitol 10.0 g/L+sucrose 30.0 g/L+agar 7.0 g/L. The pH value was 5.8.

Awnless triticale lines (T8003, T8004) which produced more callus were selected, and the callus with a diameter of more than 1 mm were transferred to 4 kinds of MS differentiation medium, and 4 calli were placed in each dish, and the petri dishes were placed in an incubator set at 27 °C with a 14-h light (3,000 Lx): 10-h dark cycle, and the medium was refreshed every 15 days. The number of green plantlets and albino plantlets was counted after 90 days. Since T8001, T8002, and T8005 lines induced fewer callus, callus from these three lines were inoculated only on D1 differentiation medium.

Plantlets acclimatization

When the regenerated green plantlets grew to 4 leaves, the parafilm of the culture bottle was removed, and 150 mL tap water was added to culture bottle, and the culture bottle was remained in the incubator set at 27 °C with a 14-h light (3,000 Lx): 10-h dark cycle for 3 days. After that, the green plantlets were transplanted into plastic pots (10 cm × 10 cm) (Jiesheng Co., Shenzhen, Guangdong, China) containing nutrient soil (Luneng Co., Tianzhu, Gansu, China). These plastic pots was placed in an artificial climate chamber set at 25 °C with a 14-h light : 10-h dark cycle.

Ploidy level analysis of the regeneration green plantlets

The ploidy levels of regenerated plants was determined by the CyFlow Ploidy Analyzer (CyFlow Cube 6; Sysmex Co., Hamburg, Germany). Briefly, one leaf were collected from each plant and then placed in 400 μL extraction buffer (CyStain UV Precise P Kit; Sysmex Co., Hamburg, Germany). The leaf was minced for 1 min using a sharp

blade and passed through a 30 μm filter to remove cell debris. Next, 1,600 μL DAPI staining solution was added to stain the nuclei prior to the analysis using the CyFlow Ploidy Analyzer (Sysmex Co., Hamburg, Germany). More than 3,000 nuclei were detected per sample. Hexaploid triticale variety 'Gannong No.3' (provided by Gansu Agricultural University, Lanzhou, Gansu, China) was used as a control (CK1) to determine whether the regenerated plants were haploid plants.

Chromosome doubling, plantlets acclimatization, and transplanting

When the green plantlets had three tillers (or were approximately 12 cm tall), they were removed from the plastic pot, rinsed, and then their roots were soaked in a solution containing 0.1% colchicine, 2% dimethyl sulfoxide, and 0.05% Tween-20 for 4.5 h chromosome doubling step. The roots were rinsed by tap water for 2 h and then the green plantlets were replanted in plastic pots and incubated in the artificial climate chamber for 3 ~ 5 weeks to resume growth. They were subsequently transferred to a field.

Ploidy level analysis of the regenerated plantlets after chromosome doubling

Flow cytometry was used to analyze the ploidy of plantlets that grew normally in the field (the booting stage). The analysis method is the same as 2.7. The octaploid tritcale variety 'Jinsong 49' (provided by the Hebei Academy of Agriculture and Forestry Science, Shijiazhuang, Hebei, China) was used as a control (CK2) to determine whether the regenerated plantlets were doubled plants.

Agronomic trait analysis

The following traits of the regenerated plants were examined at the flowering stage: plant height. The following spike parameters were examined at the dough stage: tip awn length, side awn length, spike length, number of spikelets, number of grains per spike, and grain weight per spike. The parent T2021-8003 was used as the control group (CK3).

Statistical analysis

Each petri dish with inoculated anthers was taken as a replicate, and each treatment contained 6 ~ 8 petri dishes (replicates). In plant differentiation trials, there are at least 3 replicates per treatment. The analysis of variance was performed by SPSS 20.0 software. If significant differences were detected, Duncan's multiple comparison test was performed to compare the differences. Callus induction rate (CIR), green plantlet differentiation frequency (DFG), albino plantlet differentiation frequency (DFA), green plantlet production (PRG), albino plantlet production (PRA), and plant regeneration rate (PRR) were calculated as follows.

$$\text{CIR}\ (\%) = \text{Number of calli}/\text{Number of anthers used for the inoculation} \times 100$$

$$\text{DFG}\ (\%) = \text{Number of green plantlets}/\text{Number of calli} \times 100$$

$$\text{DFA}\ (\%) = \text{Number of albino plantlets}/\text{Number of calli} \times 100$$

$$\text{PRG}\ (\%) = \text{Number of green plantlets}/\text{Number of anthers used for the inoculation} \times 100$$

$$\text{PRA}\ (\%) = \text{Number of albino plantlets}/\text{Number of anthers used for the inoculation} \times 100$$

$$\text{PRR}\ (\%) = \text{Total numbers of plantlets}/\text{Number of anthers used for the inoculation} \times 100$$

Results

Microscopic examination of anthers

The leaf sheath of the triticale branches was peeled off to expose the young spikes inside. Then, two anthers were collected from each young spike, crushed on a glass slide, stained with 1–2 drops of acetic acid magenta dye, and then covered with a cover slip. The microspores of young spikes of five triticale lines were observed under a light microscope (Fig. 2). In all the lines, most of the microspores were in the late uninucleate stage, which is the optimum stage for anther culture (Fig. 2a, b).

Analysis of variance

Table 5 showed that the significant or very significant differences were detected for triticale line, except for the DFG, PRG and PRA. The significant or very significant differences were detected for hormone concentration of induction / differentiation medium, except for the DFA, PRA and PRR, and the significant or very significant differences were detected for the interactive effects of triticale line and hormone concentration of induction / differentiation medium, except for the PRA. The multiple comparison test was completed for the above parameters with significant or very significant differences (Table 5).

Effects of triticale genotype and hormone concentrations in the medium on callus induction

Average callus induction rate among five triticale lines

There were significant differences in the average callus induction rate (CIR) among the various triticale lines (Fig. 3). The average CIR was highest in T8004 (16.67%), which were significantly higher than those of T8001, T8002 and T8005 ($P<0.05$). The lowest average CIR was in T8001 at 6.25%, which was significantly lower than those of T8003 and T8004 ($P<0.05$). These findings show that the average CIR differed significantly among the five triticale lines.

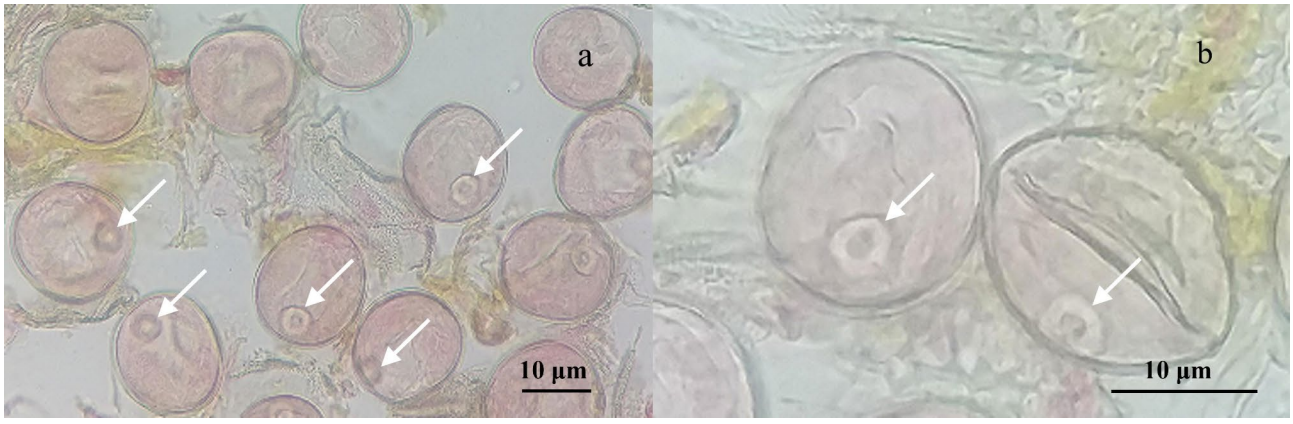

Fig. 2 Micrographs of triticale microspores at late uninucleate stage. (**a**) Microspores of T8003 line at 1000× magnification. (**b**) Microspores of T8003 line at 2000× magnification. Note The arrow pointed to the location of the nucleus of the microspore

Table 5 Variance analysis of induction and differentiation indicators of awnless triticale anther culture

Variation	F Value					
	CIR (%)	DFG (%)	DFA (%)	PRG (%)	PRA (%)	PRR (%)
Triticale line	7.13**	2.88*	1.90ns	2.21ns	0.99ns	3.49*
Hormone concentration of induction / differentiation medium	4.00*	15.48**	1.13ns	4.43*	1.13ns	1.67ns
Triticale line × hormone concentration of induction / differentiation medium	31.52**	8.27**	3.15*	3.00*	1.60ns	2.71*

Note: CIR: Callus induction rate. DFG: Green plantlet differentiation frequency; DFA: Albino plantlet differentiation frequency. PRG: Green plantlet production; PRA: Albino plantlet production; PRR: Plant regeneration rate; * indicates significant differences at 0.05 level; ** indicates significant differences at 0.01 level, NS indicates no significant; the same as below

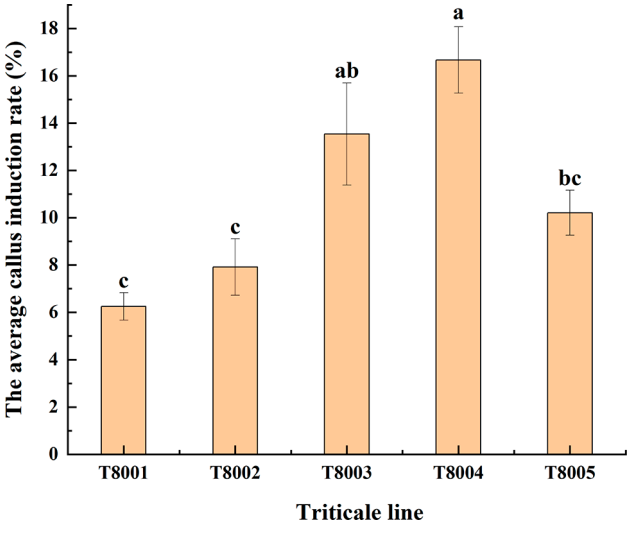

Fig. 3 Average callus induction rate in triticale lines for different hormone concentrations

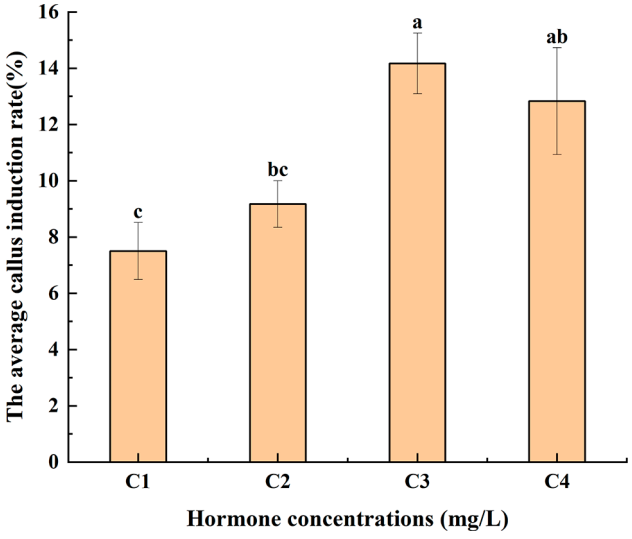

Fig. 4 Average callus induction rate in hormone concentrations for different triticale line. Note C1: 1.5 mg/L 2,4-D + 1.0 mg/L KT; C2: 1.5 mg/L 2,4-D + 1.5 mg/L KT; C3: 2.0 mg/L 2,4-D + 1.5 mg/L KT; C4: 2.5 mg/L 2,4-D + 1.5 mg/L KT

The average CIR among media with different hormone concentrations

As the 2,4-D concentration in the medium increased, the average CIR of different triticale lines first increased and then decreased. The average CIR was highest on C3 medium (14.17%), significantly higher than that of other medium, except for C4 medium. The average CIR was lowest on C1 medium (7.50%), which was significantly lower than those on C3 and C4 media. Thus, the highest average CIR was achieved on C3 medium (Fig. 4).

Significance of genotype × hormone concentration interaction for CIR

As the hormone concentration in the medium increased, the CIRs of triticale lines T8001, T8002, T8004 and

T8005 first increased and then decreased, while the CIR of T8003 gradually increased. For the genotypes T8001, T8004, and T8005, the highest CIRs were achieved on C3 medium (7.50%, 25.83%, and 13.33%, respectively), and these rates were significantly higher than those on other media. The CIR of T8002 was significantly higher on C2 medium (13.33%) than on other media, and the CIR of T8003 was significantly higher on C4 medium (21.67%) than on other media. These results indicate that the different triticale lines required different hormone concentrations to achieve the highest CIRs (Fig. 5).

Differentiation of triticale calli obtained in anther culture

Differences in differentiation frequency, plantlet production, and regeneration rate among triticale lines

Large numbers of calli were produced by the T8003 and T8004 lines, whereas fewer were produced by the T8001, T8002 and T8005 lines. To compare the effects of different hormone concentrations on callus differentiation, the calli produced by the T8003 and T8004 lines were cultured on D1, D2 and D3 differentiation media, and the calli produced by T8001, T8002, and T8005 were cultured on D1 differentiation medium. As shown in Table 6, there were significant differences in the green plantlet differentiation frequency (DFG) and plant regeneration rate (PRR) of the triticale lines among the three media containing different hormone concentrations. The highest average DFG was in T8005 (20.83%), and this was higher or significantly higher than that of other triticale lines ($P<0.05$). The highest average PRR was in T8001 (2.92%), significantly higher than that of T8002 ($P<0.05$), but not significantly different from those of T8003, T8004 and T8005 (Table 6).

Differences in differentiation frequency, plantlet production among three different differentiation media

As shown in Table 7, there were significant differences in the average DFG and PRG of the triticale lines among the three differentiation media with different hormone concentrations. The highest average DFG and PRG (29.17% and 2.43%, respectively) were achieved on D2 medium, and were significantly higher than their respective values on D1 and D3 media. These results show that the highest DFG and PRG were achieved on D2 medium, so this medium was most conducive to obtaining green plantlets from calli produced in anther culture.

Effects of the genotype × hormone interaction in terms of differentiation frequency, plantlet production, and plant regeneration rate.

The effect of the genotype × hormone concentration interaction was significant for DFG and DFA ($P<0.05$). The calli of T8003 and T8004 were cultured on D1, D2, and D3 media, and as the 6-BA concentration in the medium increased, the DFG of T8003 and T8004 showed a trend of first increasing and then decreasing. The DFG of T8003 and T8004 were highest on D2 medium (25.00% and 33.33%, respectively), and these values were higher or significantly higher than those on D1 and D3 media. The DFA of T8003 and T8004 were lowest on D2 medium (0.00% and 4.17%, respectively), and these values were lower or significantly lower than those of the same genotypes on D1 and D3 media. These results show that D2 medium was the most conducive to obtaining green plantlets of the triticale lines T8003 and T8004 (Table 8).

When the calli of the five triticale lines were cultured on D1 differentiation medium, there were significant

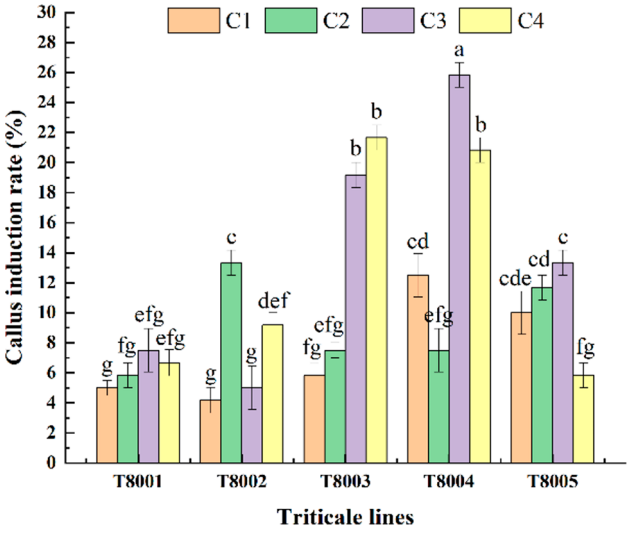

Fig. 5 Effects of the interaction between triticale line and hormone concentration on callus induction rate. Note C1: 1.5 mg/L 2,4-D + 1.0 mg/L KT; C2: 1.5 mg/L 2,4-D + 1.5 mg/L KT; C3: 2.0 mg/L 2,4-D + 1.5 mg/L KT; C4: 2.5 mg/L 2,4-D + 1.5 mg/L KT

Table 6 Average DFG and PRR in triticale lines for different hormone concentrations

Triticale line	DFG (%)	PRR (%)
T8001	15.28 ± 1.39a	2.92 ± 0.42a
T8002	0.00 ± 0.00b	0.83 ± 0.42b
T8003	16.67 ± 2.69a	1.67 ± 0.42ab
T8004	19.44 ± 4.05a	1.81 ± 0.30ab
T8005	20.83 ± 4.17a	2.50 ± 0.27a

Notes DFG: green plantlet differentiation frequency; PRR: plant regeneration rate. Different letters indicate significant difference among triticale lines ($P<0.05$). The same as below

Table 7 Average DFG and PRG in hormone concentrations for different triticale line

Treatment	Hormone concentration	DFG (%)	PRG (%)
D1	1.0 mg/L IAA + 1.0 mg/L 6-BA	9.72 ± 2.02c	1.08 ± 0.24b
D2	1.0 mg/L IAA + 1.5 mg/L 6-BA	29.17 ± 2.64a	2.43 ± 0.5a
D3	1.0 mg/L IAA + 2.0 mg/L 6-BA	18.75 ± 2.8b	1.39 ± 0.23b

Notes DFG: Green plantlet differentiation frequency; PRG: Green plantlet production. Different letters indicate significant difference among triticale lines ($P<0.05$)

Table 8 DFG and DFA of different triticale lines treated with 1 mg/IAA and varying 6-BA concentrations

Triticale line	NHC	IAA (mg/L)	6-BA (mg/L)	NC	NGP	NAP	DFG (%)	DFA (%)
T8001	D1	1.0	1.0	32	5	2	15.28 ± 1.39bc	5.55 ± 2.78b
T8002	D1	1.0	1.0	24	0	2	0.00 ± 0.00d	8.33 ± 4.17b
T8005	D1	1.0	1.0	24	4	0	16.67 ± 4.17bc	0.00 ± 0.00b
T8003	D1	1.0	1.0	20	2	1	8.33 ± 4.17 cd	4.17 ± 4.17b
	D2	1.0	1.5	20	5	0	25.00 ± 0.00ab	0.00 ± 0.00b
	D3	1.0	2.0	20	3	2	16.67 ± 4.17bc	8.33 ± 4.17b
T8004	D1	1.0	1.0	20	2	4	8.33 ± 4.17 cd	20.83 ± 4.17a
	D2	1.0	1.5	20	7	1	33.33 ± 4.17a	4.17 ± 4.17b
	D3	1.0	2.0	20	4	1	20.83 ± 4.17b	4.17 ± 4.17b

Note NHC: Number of hormone concentration; NC: Number of callus; NGP: Number of green plantlet; NAP: Number of albino plantlet; DFG: Green plantlet differentiation frequency; DFA: Albino plantlet differentiation frequency

Table 9 PRG, PRA, and PRR of different triticale lines treated with 1 mg/IAA and varying 6-BA concentrations

Triticale line	NHC	IAA (mg/L)	6-BA (mg/L)	NA	NGP	NAP	PRG (%)	PRA (%)	PRR (%)
T8001	D1	1.0	1.0	240	5	2	2.08 ± 0.42ab	0.83 ± 0.42ab	2.92 ± 0.42ab
T8002	D1	1.0	1.0	240	0	2	0.00 ± 0.00c	0.83 ± 0.42ab	0.83 ± 0.42d
T8005	D1	1.0	1.0	240	4	0	1.67 ± 0.42ab	0.00 ± 0.00b	1.67 ± 0.42bcd
T8003	D1	1.0	1.0	240	2	1	0.83 ± 0.42bc	0.42 ± 0.42ab	1.25 ± 0.72 cd
	D2	1.0	1.5	240	5	0	2.08 ± 0.42ab	0.00 ± 0.00b	2.08 ± 0.42abcd
	D3	1.0	2.0	240	3	2	1.25 ± 0.00abc	0.83 ± 0.42ab	2.08 ± 0.42abcd
T8004	D1	1.0	1.0	280	2	4	0.83 ± 0.42bc	1.53 ± 0.50a	2.36 ± 0.14abc
	D2	1.0	1.5	280	7	1	2.78 ± 0.97a	0.42 ± 0.42ab	3.19 ± 0.56a
	D3	1.0	2.0	280	4	1	1.53 ± 0.50abc	0.42 ± 0.42ab	1.94 ± 0.37abcd

Note NHC: Number of hormone concentration; NA: Number of anther; NGP: Number of green plantlet; NAP: Number of albino plantlet; PRG: Green plantlet production; PRA: Albino plantlet production; PRR: Plant regeneration rate

differences in DFG and DFA among the five lines ($P<0.05$). The DFG of T8005 was the highest (16.67%), which was significantly higher than that of T8002, but not significantly different from the other triticale lines. The T8005 genotype had the lowest DFA (0.00%), followed by T8003 (4.17%), whereas T8004 had the highest DFA (20.83%), which was significantly higher than those of the other genotypes. In summary, there were significant differences in the differentiation rate of calli among different genotypes of triticale on the same medium, and this was related to genotype (Table 8).

As shown in Table 9, the PRG and PRR of triticale plants differed significantly among the three media with different hormone concentrations ($P<0.05$). When the calli of T8003 and T8004 were cultured on D1, D2, and D3 differentiation media, the highest PRG for the calli of both T8003 and T8004 was on D2 medium (2.08% and 2.78%, respectively). The PRG of T8003 and T8004 calli on D2 medium was higher or significantly higher than those on D1 and D3 media, and the PRA of T8003 and T8004 calli was lower on D2 medium than on D1 and D3 media. These results show that D2 differentiation medium was more suitable than the D1 and D3 media for callus differentiation of the T8003 and T8004 lines (Table 9).

When all five triticale lines were cultured on D1 differentiation medium, the PRG, PRA, and PRR differed significantly among the five lines. T8001 had the highest PRG (2.08%), followed by T8005, and T8002 had the lowest PRG (0.00%). In terms of PRA, T8005 had the lowest PRA (0.00%), which was lower or significantly lower than that of other genotypes, while T8004 had the highest PRA (1.53%), which was higher or significantly higher than those of the other genotypes. In terms of PRR, T8001 had the highest PRR (2.92%), followed by T8004 (2.36%), whereas T8002 had the lowest PRR (0.83%), which was significantly lower than those of the other genotypes (Table 9). These results indicate that there were significant differences in the PRG and PRA among different triticale lines (genotypes) under the same culture conditions (Table 9).

Identification of the ploidy level of regenerated plants

During the process of anther culture, simple microspores are induced to form callus, which then differentiates into green plants. The cells of the anther wall, anther septum, and anther filament may also dedifferentiate to form callus, which can also differentiate into green plants. The green plants induced from simple microspores are haploid (triploid in this experiment), and the green plants induced from the cells of the anther wall, anther septum,

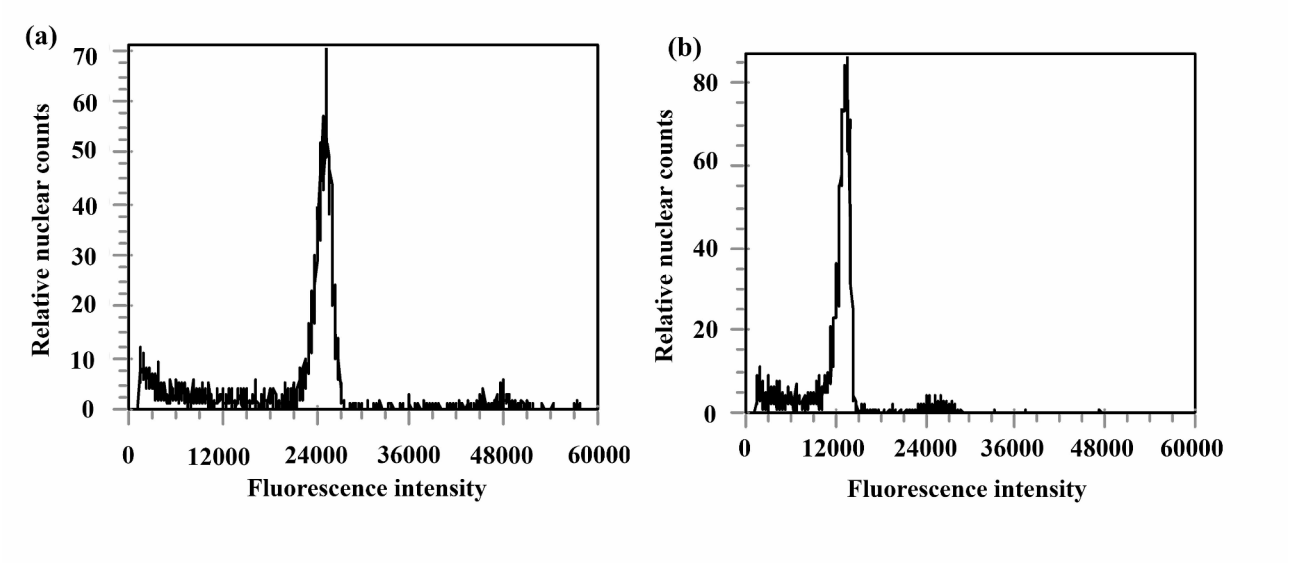

Fig. 6 Ploidy identification of triticale regenerated plantlet. Note: (**a**): 'Gannong No. 3' triticale (CK_1); (**b**): haploid regenerated plants of T8004

Table 10 The number of survival green plantlet after chromosome doubling and survival rate after chromosome doubling

Triticale line	TNGP	NHGP	NSGPCD	SR/%
T8001	5	4	3	75.0
T8002	0	0	0	0.0
T8003	10	10	9	90.0
T8004	13	11	9	81.8
T8005	4	2	2	100.0
Total	32	27	23	85.2

Note: TNGP: Number of surviving green plantlets; NHGP: Number of haploid green plantlet; NSGPCD: Number of green plants surviving after chromosome doubling; SR: Survival rate

and filament are diploid (hexaploid in this experiment). In addition, spontaneous doubling of green plants may occur during the differentiation of calli into green plants. Therefore, we analyzed the ploidy levels of the green plants obtained in anther culture by flow cytometry to distinguish between the haploids and diploids.

In this experiment, the control 'Gannon No.3' triticale (CK1) was hexaploid (2n=6x=42), and T8001, T8002, T8003, T8004, and T8005 were also hexaploids (2n=6x=42). As shown in Fig. 6, the position of the fluorescence peak in the control 'Gannon No.3' triticale (CK1) was at 21,000–27,000 (Fig. 6a). Therefore, when analyzing the green plants obtained in anther culture, a fluorescence peak at half of this value indicated that the plant was haploid. The fluorescence peak positions of green plants induced from the T8001, T8002, T8003, T8004, and T8005 lines were at 9000–15,000, approximately half of that in the control group, indicating that the induced green plants were haploids (Fig. 6b).

Chromosome doubling of haploid plants

As shown in Table 10, a total of 23 plants survived after root soaking, with survival rates ranging from 75.0 to 100% among the five genotypes. The average survival rate across the five lines was 85.2%. The survival rate of T8005 was 100%, whereas 1, 1, and 2 plants of T8001, T8003, and T8004, respectively, did not survive. The survival rates of the T8001, T8003, and T8004 lines were 75.0%, 90.0%, and 81.8%, respectively (Table 10).

Identification of doubled regenerated plants

The ploidy level of regenerated plants that grew normally to the booting stage was identified by flow cytometry. CK2 was the octaploid 'Jinsong 49' triticale (2n=8x=56) (Fig. 7a), with a fluorescence peak at 21,000–27,000. Therefore, when analyzing the green plants obtained in anther culture, a fluorescence peak at three-quarters of this value indicated that the plant was double haploid. The regenerated plants had fluorescence peaks at 16,000–21,000 (i.e., three-quarters of CK2), so they were identified as doubled haploid plantlets (Fig. 7b).

Of the 23 plants transplanted into the field, 20 survived. Of those, 15 plants were doubled haploid plants and five were mixoploid. The chromosome doubling success rate ranged from 25.0 to 63.6% among the five lines, and the average chromosome doubling success rate was 55.6%. T8004 had the highest chromosomal doubling success rate (63.6%) and T8001 had the lowest (25.0%). This difference may be related to genotype. The above results show that the chromosomal doubling success rate differed among the five genotypes of triticale (Table 11).

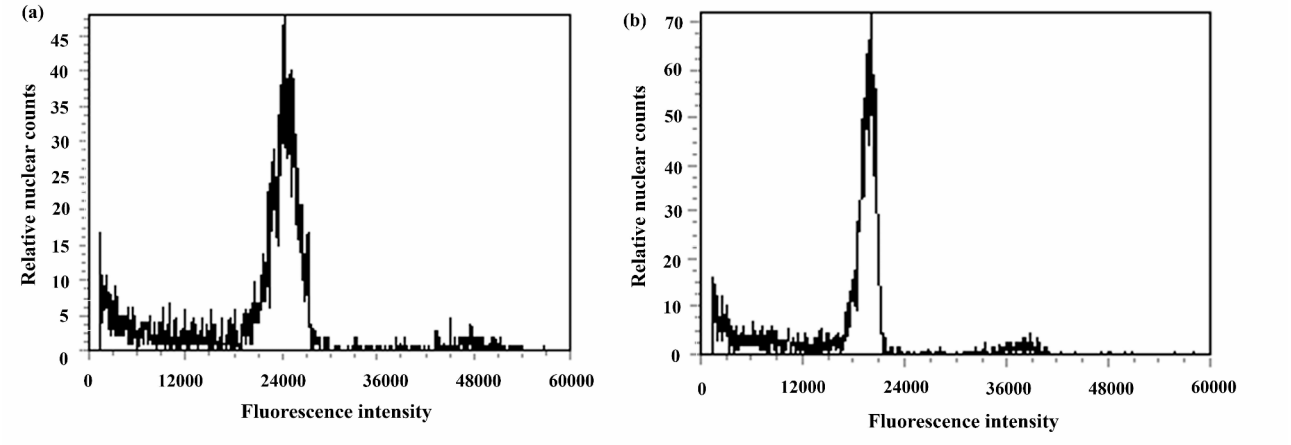

Fig. 7 Ploidy identification of octaploid triticale 'Jinsong No.49' (CK2) (**a**) and double haploid plantlet of T8004 (**b**)

Table 11 Ploidy changes and success rate of chromosome doubling in regenerated plantlet after chromosome doubling

Triticale line	NHGP	NGPT	NSGP	Double haploid plant	MOPP	SRCD/%
T8001	4	3	3	2	1	25.0
T8002	0	0	0	0	0	0.0
T8003	10	9	8	5	3	30.0
T8004	11	9	8	7	1	63.6
T8005	2	2	1	1	0	50.0
Total	27	23	20	15	5	55.6

Note: NHGP: Number of haploid green plantlet; NGPT: Number of green plantlet transplanted in field; NSGP: Number of survival green plantlet; MOPP: Mixoploidy and other ploidy plants; SRCD: Success rate of chromosome doubling

Investigation of agronomic traits in the field of DH1 generation plants

The calli that formed in anther culture differentiated to form haploid green plants, which were transplanted into the field after chromosome doubling. The agronomic traits of the doubled haploid regenerated plants that grew to the flowering stage in the field were measured (Fig. 8). To determine whether tissue culture affected various plant characteristics, the traits of the DH1 generation population were measured. As shown in Table 12, the plant height of 11 triticale plants in the DH1 generation was 126.8–153.7 cm, and the plant height of four of those plants was significantly higher than that of CK3 (T2021-8003). The tip awn and side awn of 11 regenerated DH1 plants were extremely short, both with lengths shorter than 5.0 mm, which reached the standard of awnless triticale. Thus, these 11 plants were identified as potential materials for further breeding of awnless varieties (Fig. 9). In terms of the panicle traits, the spikelet number was higher in four DH1 plants than in CK3, and the number of grains per spike was higher in two DH1 plants than in CK3. The grain weight per spike was lower in all 11 DH1 plants than in CK3. These results show that the values of the spike traits, especially grain weight per spike, were lower in the DH1 generation plants than in the control.

Discussion

Effect of genotype on callus induction rate and green plantlet differentiation frequency

The efficiency of anther culture is affected by various factors, among which the genotype of the plant is the most important [19]. Even when the culture conditions are exactly the same, the efficiency of anther culture differs among different plant genotypes [20]. High-response and low-response genotypes show quite different responses in anther culture [28]. For example, Żur et al. [25] detected a significant difference in the number of calli produced in anther culture between four 'responsive' triticale lines and four 'recalcitrant' triticale lines. Under the same culture conditions, the number of calli produced reached 60–125 per 100 anthers in the four 'responsive' lines, but only 5–12 per 100 anthers in the four 'recalcitrant' lines, demonstrating large differences in the CIR between 'responsive' and 'recalcitrant' lines. Sun et al. [29] used six genotypes of winter triticale in anther culture, and obtained CIRs ranging from 2.12% (in the winter triticale variety H311) to 14.88% (in the winter triticale variety H11). These findings highlight the importance of genotype in anther culture of triticale. In anther culture of two winter wheat varieties, the high-response wheat genotype 'Svilena' produced an average of 179.9 calli per 100 anthers, whereas the low-response wheat genotype

Fig. 8 Production of doubled haploid triticale plants. **a**: Collected triticale young spikes; **b**: The late uninucleate microspores; c: Induction medium inoculated with anthers; **d**: Callus; **e**: Differentiated green plantlet and albino plantlet; **f**: Growing green plantlet; **g**: Open the culture bottle and train seedling for 3 days; **h**: Green plantlets before chromosome doubling; **i**: Green plantlets before chromosome doubling. **j**: Green plantlets grew for 2 weeks after chromosome doubling; **k**: Green plantlets grew in a greenhouse

'Berengar' produced only 4.8 calli per 100 anthers [30]. In anther cultures of oat [31], wheat [32], and rye [33], genotype also significantly affected the efficiency of callus formation. In this study, under the same induction conditions (C3 medium), the CIR varied widely among the five genotypes of triticale (range, 5.00–25.83%). The results are consistent with those of Żur et al. [25] and Sun [29], and illustrate the importance of genotype in the success of anther culture. In this study, the CIR of five winter triticale lines ranged from 5.00 to 25.83%; higher than the rates reported by Sun (2.12–14.88%) and those of the four 'recalcitrant' lines reported by Żur et al. (5–12%), but lower than those of the four 'responsive' triticale lines reported by Żur et al. (60–125%). Therefore, the next step should be to find high-response genotypes of awnless triticale.

As well as affecting the CIR, the genotype of the materials also affects the DFG of triticale. Immonen and Robinson [34] studied the regeneration ability of 10 genotypes of winter triticale from Canada and Belarus. They found that after 2 weeks of cold treatment, each genotype of triticale produced green plantlets, but there was a significant difference in the DFG (0.0–26.7%) among the 10 genotypes. The DFG was highest in the 'Paljus' line (26.7%), followed by 'LP4496.5.92' (24.7%), and lowest in the 'Modul' line (3.1%). Ślusarkiewicz-Janzina and Ponitka (2003) [35] used seven triticale genotypes and obtained DFGs ranging from 0.4–7.2%. Among those

Table 12 The agronomic traits of the 11 DH1 generation double haploid plants

Number	Plant height/cm	Tip awn/mm	Side awn/mm	Spike length/cm	Number of spikelet	Number of grains per spike	Grain weight per spike/g
CK3	142.5 ± 0.9d	3.51 ± 0.3 cd	2.75 ± 0.2abc	12.5 ± 0.3a	30.3 ± 0.3 cd	42.7 ± 1.5b	1.99 ± 0.12a
Z23-1	126.8 ± 0.8 g	3.6 ± 0.1bcd	2.74 ± 0.2abc	11.2 ± 0.2de	31.0 ± 1.0bc	43.0 ± 1.0b	1.50 ± 0.07 fg
Z23-2	134.1 ± 0.7ef	3.24 ± 0.1d	3.07 ± 0.1abc	11.7 ± 0.3bcd	34.7 ± 0.7a	49.7 ± 1.5a	1.67 ± 0.08bc
Z23-3	151.7 ± 1.3ab	4.26 ± 0.1ab	3.89 ± 0.4a	12.3 ± 0.1ab	33.3 ± 0.7ab	41.0 ± 1.2bc	1.79 ± 0.10cde
Z23-4	135.8 ± 1.2ef	3.49 ± 0.3 cd	2.63 ± 0.5bc	10.6 ± 0.3e	26.7 ± 0.7e	28.7 ± 1.3e	1.40 ± 0.03de
Z23-5	144.7 ± 0.8 cd	4.26 ± 0.2ab	2.02 ± 0.5c	11.5 ± 0.3 cd	28.0 ± 1.2de	32.3 ± 1.5de	1.19 ± 0.05bcd
Z23-6	133.0 ± 1.2f	3.68 ± 0.2bcd	2.07 ± 0.1c	9.5 ± 0.1f	28.7 ± 0.7cde	35.0 ± 1.7d	1.35 ± 0.05ab
Z23-7	153.7 ± 0.7a	2.94 ± 0.5d	3.96 ± 0.1a	12.3 ± 0.1ab	33.3 ± 0.7ab	44.0 ± 2.1b	1.87 ± 0.04efg
Z23-8	150.3 ± 0.7b	3.39 ± 0.2 cd	2.63 ± 0.5bc	12.0 ± 0.2abc	30.7 ± 1.3 cd	37.0 ± 1.5 cd	1.48 ± 0.07 g
Z23-9	146.5 ± 0.9c	4.50 ± 0.2a	2.96 ± 0.4abc	11.7 ± 0.1bcd	29.3 ± 0.7 cd	34.0 ± 1.0d	1.26 ± 0.06efg
Z23-10	137.0 ± 1.2e	4.11 ± 0.1abc	3.87 ± 0.1a	10.7 ± 0.3e	30.7 ± 0.7 cd	46.0 ± 2.6ab	1.73 ± 0.07ab
Z23-11	144.3 ± 1.6 cd	4.73 ± 0.2a	3.79 ± 0.6ab	11.0 ± 0.2de	28.7 ± 0.7cde	37.0 ± 1.5 cd	1.55 ± 0.06def

Fig. 9 Spike traits of awnless triticale plants Z23-8(**a**) and Z23-11(**b**) in the DH1 generation

seven genotypes, '19Sz' showed the highest DFG (7.2%), followed by '4Sz' (4.3%), and '16Ch' had the lowest DFG (0.4%). The results of those studies clearly show that genotype has a significant effect on the DFG. In the present study, when the five genotypes were cultured on the same induction medium (D2), T8004 had the highest DFG (33.3%). This value was significantly higher than the highest DFG values reported by Immonen and Robinson [34], and Ślusarkiewicz-Jarzina and Ponitka [35], and these differences in values are related to triticale genotype. Our results indicate that T8004 and T8003 have strong differentiation abilities and produce high-quality green plantlets. Therefore, both of these lines are suitable materials for further research on anther culture of triticale.

Effect of hormone concentration ratio on callus induction rate and green plantlet differentiation frequency

In anther culture, hormones are important factors for callus induction and callus differentiation, and the type and concentration of exogenous hormones added to the culture medium will have an important impact on the induction and proliferation of calli or embryoids [35]. Auxins such as 2,4-D, indole acetic acid (IAA), indole-3-butyric acid (IBA), and NAA, and cytokinins such as 6-BA, KT, and ZT (zeatin) are commonly used hormones [36, 37]. In triticale anther culture, the auxin 2,4-D plays an important role in inducing callus formation, but its effects are weaker if it is used alone [38]. Although the addition of only 2,4-D to MS medium induced callus formation in red clover, few calli were produced and their quality was poor, and this was not conducive to the differentiation of buds and roots in the later stages [39]. Similar findings have been reported for wheat in anther

culture [40]. Therefore, 2,4-D should be used with a cytokinin at an appropriate concentration to induce the formation of abundant high-quality calli.

The combination of 2,4-D and KT is often used to induce callus from plant materials. Sun [29] conducted triticale anther culture with different types and ratios of hormones and obtained the highest CIR (14.88%) on medium containing 2.0 mg/L 2,4-D+1.0 mg/L KT. Żur et al. [25] found that C17 medium containing 2.0 mg/L 2,4-D+0.5 mg/L KT was the most suitable for inducing calli from four triticale lines ('DH19', 'DH72', 'DH119', and 'DH144'), obtaining CIRs of up to 12.0%. The types and ratios of exogenous hormones have also been shown to affect the CIR in anther cultures of other crops. For example, Barroso et al. [41] conducted anther culture using eight varieties of capsicum (G3–G10) and obtained CIRs of 1.0–8.6%. The highest CIR of 8.6% (in the G3 genotype), was obtained by culturing the anthers on C medium supplemented with 22.6 μm 2,4-D+23.25 μm KT. In this study, the anthers of five genotypes of triticale were cultured on C3 induction medium (2.0 mg/L 2,4-D+1.5 mg/L KT). The T8001, T8004, and T8005 lines had the highest CIRs. These results indicate that C3 induction medium is suitable for callus induction from awnless triticale lines.

Compared with callus induction, callus differentiation requires different typnes and concentrations of hormones. The combination of IAA and 6-BA is commonly used to induce triticale calli to differentiate into plantlets. Li [42] obtained the highest DFG (25.0%) by culturing calli of triticale 'Gannong No.1' and 'Shida No.1' on MS medium containing 1.0 mg/L IAA+1.0 mg/L 6-BA. González and Jouve [43] found that the calli of 10 winter triticale lines cultured on medium containing 1.0 mg/L IAA+0.4 mg/L NAA had an average DFG of 7.77%, while the calli cultured on medium containing on 0.2 mg/L NAA+0.5 mg/L 6-BA had an average DFG of 6.67%. In the present study, the DFGs (25.00% and 33.33%, respectively) of T8003 and T8004 on D2 medium containing 1.0 mg/L IAA+1.5 mg/L 6-BA are higher than those reported by Li [42], and González and Jouve [43]. As the concentration of 6-BA in the medium increased, the DFG increased first and then decreased. This may be because 6-BA can promote bud formation, so appropriately increasing the 6-BA concentration was conducive to bud differentiation. Therefore, D2 medium was more conducive to DFG in anther cultures of T8003 and T8004.

Effect of the concentration of colchicine solution and soaking time on the success of chromosome doubling.

Colchicine treatment can improve the probability of polyploidy and increase the polyploidy induction rate of regenerated plants, but it can also negatively affect the plantlets, causing wilting and reducing the survival rate [44]. Colchicine disrupts spindle formation by inhibiting the formation of spindle filaments. This means that the replicated chromosomes cannot be pulled towards the poles and separate, resulting in the doubling of chromosomes [45]. Different concentrations of colchicine solution and different soaking times significantly affect the success rate of chromosome doubling of regenerated plants. Slusarkiewicz-Jarzina et al. [46] soaked the roots of 215 triticale haploid regenerated plants in 0.1% colchicine solution for 6 h (at 25 °C in light conditions), and obtained 128 doubled haploid plants, i.e., a chromosome doubling efficiency of 59.9%. In other crops, maize plantlets showed a chromosome doubling rate of 31.83% when their roots were soaked in 0.10% colchicine for 5.0 h, and a lower chromosome doubling rate (27.67%) when the roots were soaked with 0.70% colchicine for 5.0 h [47]. In this experiment, chromosome doubling of the regenerated plants was induced by soaking the roots in 0.1% colchicine solution for 4.5 h, and the average success rate of chromosome doubling was 55.6%. The highest chromosome doubling success rate was in T8004 (63.6%), higher than those reported by Slusarkiewicz-Jarzina et al. [46] and Chaikam et al. [47]. The chromosome doubling efficiency of the five triticale genotypes in this study ranged from 25.0 to 63.6%, which shows that the genotype also affected the efficiency of chromosome doubling, consistent with the findings of Ragot and Steen [47]. The concentration of the colchicine solution and the soaking time significantly affected the chromosome doubling efficiency and also affected the survival rate of the regenerated plantlets.

Conclusions

Hormone concentrations in the medium significantly affected the anther culture of five triticale lines. Among the five genotypes of triticale, T8004 showed the best performance in anther culture. Among the various treatments, the C3 treatment (2.0 mg/L 2,4-D+1.5 mg/L KT) had the best callus induction effect, and the D2 treatment (1.0 mg/L IAA+1.5 mg/L 6-BA) had the best plantlet differentiation effect. The different genotypes of triticale required different hormone concentrations for successful anther culture. The 11 DH1 double haploid plants all reached the standard of awnless triticale. Thus, they have potential applications as materials for breeding new awnless triticale varieties.

Abbreviations

2,4-D 2,4	Dichlorophenoxyacetic acid
KT 6	Furfurylaminopurine
DAPI 4',6	Diamidino-2-phenylindole dihydrochloride
ABA	Abscisic Acid
IAA	Indoleacetic Acid
6-BA	N6-Benzyladenine
CIR	Callus Induction Rate
DFG	Green Plantlet Differentiation Frequency

DFA	Albino Plantlet Differentiation Frequency
PRG	Green Plantlet Production
PRA	Albino Plantlet Production
PRR	Plant Regeneration Rate
DH	Double Haploid

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Author contributions

WD and JM designed the experiment. JM, FZ and YZ performed the experiment and data analysis. JM wrote the manuscript. XT and WD helped to revise the manuscript. All authors reviewed and approved the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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