Phyton-
International Journal of
Experimental Botany

Tech Science Press

http://doi.org/10.32604/phyton.2026.083697

ARTICLE

Genotype-Specific Androgenic Responses of Spring Barley Lines to Cold Pretreatment for Efficient Doubled Haploid Regeneration and Accelerated Breeding Cycles

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Received: 08 April 2026; Accepted: 01 June 2026; Published: 30 July 2026

ABSTRACT: Doubled haploid technology is an important tool for accelerating barley breeding by enabling the rapid development of fully homozygous lines. However, the efficiency of androgenesis in barley remains highly genotype-dependent and is strongly influenced by pretreatment conditions, particularly cold exposure. Despite advances in barley anther culture protocols, the optimal duration of cold pretreatment to enhance androgenic response and green plant regeneration remains poorly understood for many breeding materials. This study therefore aimed to examine the effects of genotype and cold pretreatment duration on androgen induction and regeneration efficiency in spring barley lines. Fifteen barley lines, including the control variety rihane, were evaluated for anther culture response. Ears containing microspores at the appropriate developmental stage were subjected to two cold pretreatments at 4°C (3 and 5 weeks) before being cultured on BAC3 induction medium. The androgenic response was assessed by the induction rate, regeneration efficiency, and the frequencies of green and albino plant production. Significant variation dependent on genotype and genotype × treatment interactions was observed. Short-term cold pretreatment promoted embryogenic induction in some genotypes, notably OR 1-10, while prolonged exposure enhanced responses in others, particularly OR 1-6 and OR 1-5. Regeneration efficiency and green plant production were similarly influenced by genotype and pretreatment duration. Prolonged cold treatment improved regeneration and chlorophyllous plant production in several genotypes but reduced performance in others. Albino plant formation also varied considerably among genotypes, highlighting its persistent impact on androgenetic efficiency. These findings provide valuable insights into genotype-dependent androgenic responses and establish a basis for optimizing cold pretreatment strategies in barley anther culture. Such genotype-specific protocols could significantly enhance doubled haploid production efficiency and facilitate the rapid development of improved barley cultivars adapted to future breeding challenges.

KEYWORDS: *Hordeum vulgare* L.; anther culture; doubled haploid; genotype; cold pretreatment; *in vitro* regeneration

1 Introduction

One of the key challenges in modern plant breeding is the rapid development of new crop varieties that combine high agronomic performance with resilience to changing environmental conditions driven by emerging biotic and abiotic stresses. This necessity has become increasingly urgent due to the accelerating

global population growth, climate change, and the demand for sustainable agricultural practices [1]. To meet these challenges, plant breeders are increasingly turning to advanced plant biotechnological tools. Among them, doubled haploid (DH) technology has emerged as a powerful method to significantly reduce both the cost and the time required to produce genetically fixed lines. This is achieved by shortening the process of homozygosity fixation from the traditional 7–8 generations of selfing crosses to a single generation [2].

In cereal crops, haplodploidisation technique via androgenesis or gynogenesis plays a critical role in accelerating breeding cycles, enhancing selection efficiency, and improving trait stabilization [3]. Of these methods, androgenesis via anther or isolated microspore culture remains the most widely adopted in cereals due to its relatively higher efficiency, scalability, and cost-effectiveness [4]. DH lines, obtained via androgenesis, are fully homozygous and genetically stable, making them ideal for both cultivar development and fundamental research such as QTL mapping, genome-wide association studies, and genomic selection [5]. Furthermore, microspore culture offers novel breeding material by enabling the occurrence of spontaneous mutations and gametoclonal variation [6]. The anrogenesis process involves reprogramming immature microspores to deviate from their natural gametophytic pathway and instead follows a sporophytic development to form haploid embryos. These embryos can develop into haploid plants that are subsequently chromosome-doubled either spontaneously or by antimitotic agents such as colchicine to obtain DH individuals [7]. However, the efficiency of this process is significantly influenced by several factors, including genotype, developmental stage of microspores, culture medium composition, and pre-treatment conditions [8].

Barley (*Hordeum vulgare* L.) is one of the world's most important cereal crops, ranking fourth globally in terms of production after wheat, maize, and rice [9]. This cereal crop is one of the oldest domesticated cereal crops and has played a foundational role in the development of human civilizations. Due to its strong adaptability, especially to harsh environmental conditions, it is increasingly regarded as a promising crop for the future in the context of a rapidly changing climate and rising abiotic stresses [10]. It plays a vital role not only as a staple food and livestock feed but also as a key raw material in the malting and brewing industries. Barley is particularly valued for its adaptability to diverse and often harsh environments, including marginal soils and drought-prone areas, making it a critical crop for food security under changing climate conditions [11]. The crop's genetic diversity and wide range of agronomic traits provide an excellent basis for breeding programs aimed at improving yield, stress tolerance, and quality characteristics [12]. However, traditional barley breeding faces constraints due to its relatively long breeding cycles and complex polygenic traits, which slow the delivery of improved cultivars to farmers. Consequently, integrating advanced biotechnologies such as doubled haploid production into barley breeding is essential to accelerate genetic gains and meet current and future agricultural challenges [13].

While doubled haploid (DH) technology is a powerful tool for accelerating genetic improvement and varietal selection, the successful induction of androgenic embryos is a complex process frequently limited by developmental arrest prior to plantlet formation. Androgenetic effectiveness depends on a precisely balanced synergy between several critical factors, including the microspore developmental stage, the phytohormonal composition of the induction medium, and explant pretreatments [14]. Stress treatment methods such as cold stress, heat shock, and starvation are frequently employed to enhance microspore reprogramming and promote embryogenic induction. Additionally, the efficiency of androgenesis is often constrained by species-specific and genotype-specific responses, necessitating the optimization of protocols tailored to the genetic background of the target crop [6].

In order to develop high-performing barley varieties adapted to semi-arid environments, the present study evaluates the androgenic response of fifteen spring barley genotypes through anther culture. The aim is to identify genotype specific differences in androgenic potential and assess the effect of cold pretreatment on embryo induction and plant regeneration, with the ultimate goal of producing DH plants. This work focuses on the screening of introduced lines for their anther culture suitability. Anther culture response will be an additional criterion for selecting parents for directed crosses to accelerate our breeding program using anther culture as a doubled haploids production method. The findings will contribute to the optimization of DH technology in barley breeding, supporting the development of genetically uniform and agronomically superior lines for sustainable crop improvement in challenging environments.

2 Materials and Methods

2.1 Plant Material

Fifteen barley breeding lines, including the check variety rihane, from the INRAT barley breeding program were used in this study. These lines were selected from a larger set of 150 breeding lines developed within the National Barley Breeding Program coordinated by INRAT in collaboration with ICARDA. The selected lines were cultivated under open-field conditions at the INRAT experimental station in Beja, Tunisia (Fig. 1), and were chosen based on their agronomic performance, including plant height, heading date, tillering ability, and grain production traits. These lines represent potential parental material for the barley breeding program. The variety Rihane was included as a control due to its favorable androgenic response. The characteristics of the studied barley lines are presented in Table 1.

Table 1: List and pedigree of the 15 barley lines used for anther culture.

N°	Lines	Pedigree
1	OR 1-2	LBIRAN/UNA80//LIGNEE640/3/LEGACY//PENCO/CHEVRON-BAR
2	OR 1-10	LIGNEE527/GERBEL/3/BOY-B*2/SURB//CI12225.2D/4/M104/6/LEGACY/4/TOCTE//GOB/HUMAI10/3/ATAH92/ALELI/5/ESMERALDA
3	OR 1-8	RHANE-03/3/AS46/ATHS*2//ATHS/LIGNEE686/6/M64-76/BEN//JO/YORK/3/M5/GALT//AS46/4/HJ34-80/ASTRIX/5/M6/ROBUR-35-6
4	OR 1-9	ARAR/LIGNEE527/4/GLORIA'S'/SAIDA//MTN'S'/EH165/3/LBIRAN/UNA80//LIGNEE640
5	OR 2-3	ATAHUALPA//ALANDA-01/HAMRA/3/LITANI
6	rihane	ATLAS 46/ARRIVAT//ATHENAIS
7	OR 2-13	LA MOLINA 95/4/ALELI/ESCOBA/3/ARUPO/K8755//MORA
8	OR 1-5	ALANDA/5/ATHS/4/PRO/TOLI//CER*2/TOLI/3/5106/6/BACA'S'/3/AC253//CI08887/CI05761
9	OR 2-1	ALANDA-01/4/ALANDA//LIGNEE527/ARAR/3/BF891M-612
10	OR 1-4	VMORALES P.STO/3/LBIRAN/UNA80//LIGNEE640/4/BLLU/5/PETUNIA_1
11	OR 1-6	ALANDA/HAMRA/4/AVT/ATTIKI//M-ATT-73-1/3/ATHS/LIGNEE686
12	OR 2-9	U.SASK.1766/API//CEL/3/WEEAH/4/LIGNEE527/NK1272/5/EXPRESS
13	OR 1-7	ELDORADO//ALANDA/HAMRA-01
14	OR 1-12	RHN-03/ELDORADO/5/RHN-03//LIGNEE527/NK1272/4/LIGNEE527/CHN-01/3/ALANDA
15	OR 2-2	ATHS/LIGNEE686/4/AVT/ATTIKI//ATHS/3/GIZA121/PUE

Figure 1: Barley varieties cultivated in the experimental field in Béjà.

2.2 Determination of Microspore Developmental Stage

Morphologically, the optimal stage for spike harvesting corresponds to when the spike remains enclosed within the flag leaf sheath, with the awns emerging slightly, as described by Sunderland [15]. This phenological marker typically coincides with the mid to late uninucleate development stage of microspores, which is considered the most responsive stage for androgenesis. The tillers were harvested from mother plants when the spike awns extended no more than 0.1 cm beyond the sheath, marking the onset of spike swelling and awn emergence. Only spike that predominantly exhibit microspores at the middle to late uninucleate stage were selected for culture, as verified by optical microscopy, in order to maximize embryogenic induction potential.

2.3 Cold Spike Pretreatment

Prior to *in vitro* culture, barley spikes were carefully cleaned by removing the surrounding leaf sheaths and trimming the awns to eliminate both proximal and distal anthers. The cleaned spikes were then placed upright in 15 cm glass containers, each containing a 4 cm dish of water to maintain internal humidity. The containers were sealed with Parafilm and stored at 4°C. To determine the optimal cold pretreatment duration for inducing the shift of microspore development from the gametophytic to the sporophytic pathway, two treatment periods were tested:

Treatment T1: Cold pretreatment at 4°C for 21 days (three weeks).

Treatment T2: Cold pretreatment at 4°C for 35 days (five weeks).

The selected durations (3 and 5 weeks at 4°C) were chosen based on previous studies reporting the positive effects of cold pretreatment on microspore reprogramming and androgenesis induction. In particular, a 5-week cold pretreatment at 4°C was successfully applied in durum wheat anther culture [16]. Both treatments were conducted in complete darkness, a condition known to promote microspore reprogramming and enhance embryogenic potential.

2.4 Induction Phase of Androgenesis

Anthers were cultured on a modified BAC3 induction medium, originally described by Cai [17], in whitch Ficoll was replaced by agar. The detailed composition of the medium is provided in (Table 2). It was

prepared from concentrated stock solutions containing macroelements, microelements, vitamins, organic acids, iron sources, amino acids, and maltose (60 g/L) as the primary carbon source. To support microspore development, the medium was supplemented with indole-3-acetic acid (IAA) and kinetin (0.5 mg/L each) and solidified with 8 g/L agar. After adjusting the pH to 6.2 using NaOH, the medium was sterilized by autoclaving at 120°C (1 bar) for 20 min. Post-sterilization, 30 mL of medium was dispensed into each 9 cm Petri dish.

Table 2: Composition of BAC3 induction and regeneration medium (modified).

Medium Components	BAC3 Induction Medium (mg/L)	BAC3 Regeneration Medium (mg/L)
Macro elements		
KNO_3	2600	2600
NH_4NO_3	200	200
$(NH_4)_2SO_4$	400	400
KH_2PO_4	170	170
$NaH_2PO_4{\cdot}H_2O$	150	150
$CaCl_2{\cdot}2H_2O$	600	600
$MgSO_4{\cdot}7H_2O$	300	300
Iron source		
$FeNa_2$-EDTA	40	40
Micro salts		
H_3BO_3	5	5
$MnSO_4{\cdot}4H_2O$	5	5
$CuSO_4{\cdot}5H_2O$	0.025	0.025
$CoCl_2{\cdot}6H_2O$	0.025	0.025
$KHCO_3$	50	50
$Na_2MoO_4{\cdot}2H_2O$	0.25	0.25
KI	0.8	0.8
Other inorganic components		
$KHCO_3$	50	50
$AgNO_3$	10	10
Vitamins		
Myo-inositol	2000	100
Pyridoxine HCl	1	0.5
Thiamine HCl	1	1
Nicotinic acid	0.5	0.5
Ascorbic acid	1	1
Organic acids		
Citric acid	10	10
Pyruvic acid	10	10
Carbohydrates		
Maltose	60,000	-
Sucrose	-	30,000
Growth regulators		
NAA	2	-
BAP	1	-
IAA	-	0.5
Kinetin	-	0.5
Other organic components		
Casein hydrolysate	300	300
Agar	8000	8000
pH	6.2	6.2

Prior to inoculation, barley spikes were trimmed to remove awns and surface-sterilized by immersion in a sodium hypochlorite solution for 20 min. This was followed by three successive rinses with sterile distilled water to eliminate any residual disinfectant. Under aseptic conditions in a laminar flow hood, anthers were dissected from the central part of each spike using fine sterile forceps and placed onto BAC3 induction medium. For each genotype and treatment, sixty anthers were cultured per Petri dish, with each dish serving as a single replicate (four replicates were performed per condition). The inoculated Petri dishes were incubated in a growth chamber at 27°C in complete darkness for one month. The number of responsive anthers was recorded at the end of the incubation period. The Androgenic Induction (AI) rate was calculated as the percentage of cultured anthers that successfully produced embryogenic structures (embryos or calli) relative to the total number of anthers inoculated, using the following formula:

AI (%) = (number of embryo-like structures/total number of cultured anthers) × 100

2.5 Regeneration Phase of Androgenesis

The regeneration medium was formulated based on the composition of the BAC3 induction medium, with specific modifications to the types and concentrations of growth regulators, vitamins, and the carbon source. In this phase, saccharose (30 g/L) replaced maltose as the primary carbon source. The medium was enriched with 2 mg/L of 6-benzylaminopurine (BAP) and 1 mg/L of naphthaleneacetic acid (NAA) at. The detailed composition of the regeneration medium is provided in (Table 2).

Embryos or calli previously induced on the BAC3 induction medium were carefully transferred onto Petri dishes containing the regeneration medium. These cultures were then incubated under controlled environmental conditions in a growth chamber set at 25°C, with a 16-h photoperiod to promote shoot and root development. The regeneration period lasted for one month. At the end of this phase, the number of regenerated plants was recorded. Among them, the numbers of chlorophyllous and albino plantlets were determined.

2.6 Aclimatization

The plantlets were carefully transferred to pots containing a sterile soil–peat mixture and placed in a controlled growth chamber maintained at 25°C. During the acclimatization period, humidity and light conditions were gradually adjusted to facilitate the transition from *in vitro* to *ex vitro* conditions.

2.7 Chromosome Counting

Chromosome counts were performed on actively growing root tips using the Feulgen staining technique, following the protocol described by Doré and al. [18]. The procedure included the following steps: root tips were pretreated in α-bromonaphthalene-saturated water for 2 h at room temperature (RT), then fixed in acetic alcohol at 5°C for a minimum of 2 h. This was followed by hydrolysis in 5 N hydrochloric acid for 30 min at RT. The samples were then stained with Schiff's reagent for 2 h in the dark at RT. Finally, the root tips were mounted in a drop of acetic water. Chromosomes were observed and counted under a light microscope.

2.8 Chromosome Doubling

Chromosome doubling was carried out at the three-tiller stage using colchicine treatment. Plants were carefully uprooted from their pots, and the roots were gently washed and trimmed to approximately 1.5 cm in length. A small incision was made at the base of each tiller to enhance absorption of the colchicine

solution. The plants were then immersed up to 5 cm in a 0.05% (w/v) colchicine solution for 5 h. After treatment, they were thoroughly rinsed with clean water and replanted into fresh pots.

2.9 Statistical Analysis

Statistical analyses were performed using ANOVA in SPSS version 21 to evaluate the effects of genotype and cold pretreatment on androgenesis parameters. Significant differences between treatment means were determined using Duncan's multiple range test at a significance level of 0.05. All measured variables related to androgenic response were included in the analysis. Experimental data are expressed as the mean ± standard deviation (SD) of four replicates.

3 Results

3.1 Identification of the Optimal Microspore Development Stage

The optimal stage for spike harvesting by the strong correlation between the optimal cytological stage of microspores and a distinct, easily recognizable morphological marker. Microscopic analysis of microspores stained with aceto-carmine revealed that the ideal time to harvest spikes was when they were still enclosed within the flag leaf sheath, with approximately 1 cm of the awn protruding. This morphological stage corresponded to the majority of microspores being at the early to mid-uninucleate stage, particularly in the central florets (Fig. 2a). It is important to note that microspore development varied among florets of the different barley lines studied. These complementary indicators enabled optimized sampling based on the specific genotype. Adjusting the harvest stage according to genotype proved essential for enhancing the overall efficiency of anther culture in this study. Our results further confirm that the successful induction of androgenesis in barley strongly depends on the alignment of both morphological and cytological criteria at the time of spike collection.

3.2 Variation of Androgenic Induction Potential

Initial cell divisions were first observed microscopically 4 to 5 days after culture initiation, manifesting as individual mitotic figures or small clusters of dividing cells (Fig. 2). These proliferative clusters subsequently expanded, developing into embryogenic structures. Microscopic examinations conducted at five-day intervals during the first 20 days of the induction phase confirmed the progressive microspore division and proliferation of androgenic embryo-like stuctures. The formation of these structures indicated the successful reprogramming of microspores toward the sporophytic developmental pathway. The embryo-like structures were developed from microspores on BAC3 induction medium 21–28 days after anther inoculation in T1 cold treatment and 15–20 days in T2 treatment.

By the end of the induction phase, two distinct types of structures were observed: embryos symmetrical, organized globular bodies with a smooth or slightly hairy yellowish-white surface and calli, which appeared as irregular, undifferentiated cell masses lacking defined morphology (Fig. 3). In fact, the two main routes the process of androgenesis takes are direct and indirect androgenesis. In the direct pathway (Fig. 3a–d), the microspore undergoes vegetative nucleus divisions to form a pro-embryo like structure which follows the standard stages of embryo development (globular, heart, torpedo, and cotyledonary stages). In this pathway, microspores develop directly into embryos without an intervening callus stage. More over, it minimizes the risk of genetic mutations, ensuring that regenerated plants are true to the original gametophyte genotype. Alternatively, the microspore may follow an indirect pathway by first forming an undifferentiated callus mass (Fig. 3e–h). This callus-mediated regeneration is generally less desirable as it increases the incidence of somaclonal variation and genetic instability compared to direct embryogenic development.

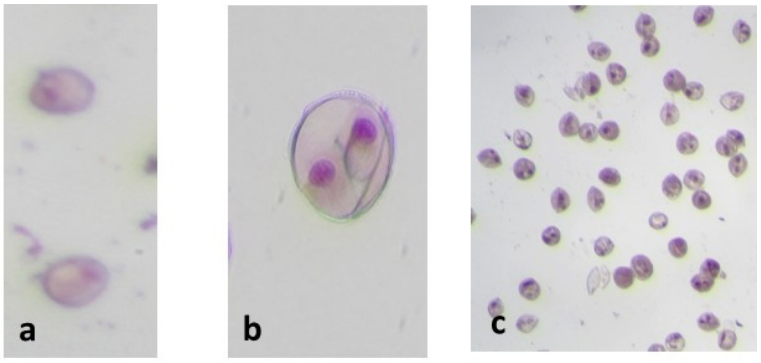

Figure 2: *In vitro* androgenesis of barley microspores; (**a**) Barley microspores in the mid uninuclear stage of development; (**b**) symmetrical division at 5 days after culture initiation 3 in BAC3 induction medium; (**c**) Near 100% dividing microspores at 7 days after culture initiation.

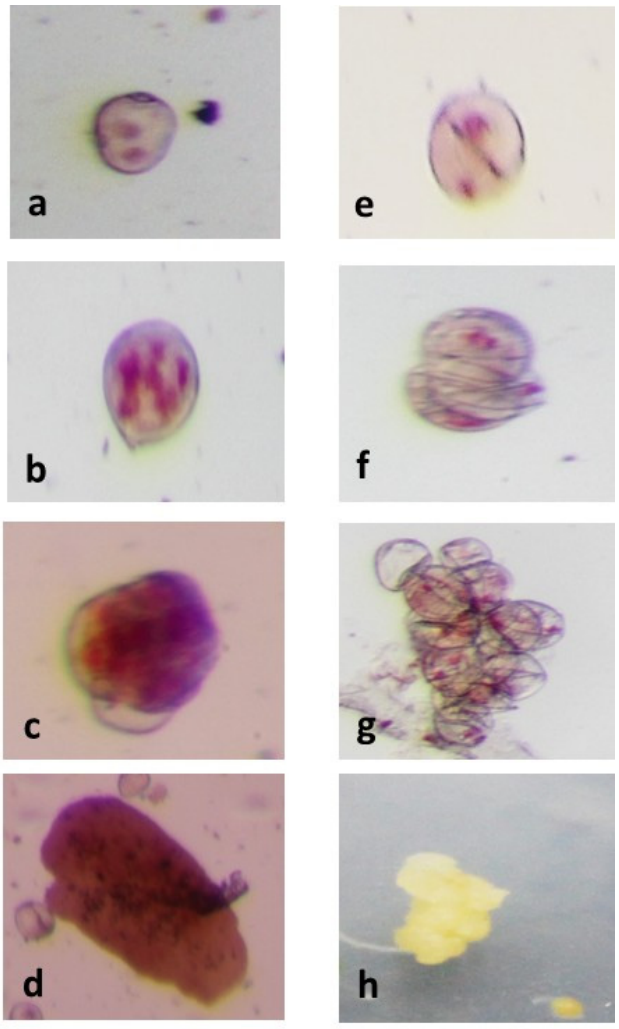

Figure 3: Microspore development pathways; (**a**–**d**) direct androgenesis pathway: the microspore behaves like a zygote and transforms directly into an embryo; (**e**–**h**) indirect androgenesis pathway: the microspores undergo irregular divisions to form a callus.

The androgenic potential of the fifteen spring barley lines, pretreated at 4°C for 21 (T1) and 35 days (T2), was assessed using the Androgenic Induction (AI) rate. Significant genotypic variations in the formation of embryogenic and callogenic structures were clearly observed across the studied genotypes. Two-way ANOVA confirmed that both the genetic background and the duration of cold stress, as well as their interaction, exerted a highly significant influence on AI efficiency (Table 3).

Table 3: Summary of two way variance analysis of the studied parameters according to treatment, line, and their interaction factors (df: degrees of freedom, F: F-statistic).

Source	Parameters	Sum of Squares	df	Mean Square	F Value	Significance
Treatment	AI	21.24	1	21.24	24.62	<0.001***
	RR1	32.70	1	32.70	4.53	0.04*
	RR2	6887.85	1	6887.85	5.05	0.03*
	GPR-A	5.60	1	5.61	1.80	0.18ns
	GPR-P	52.73	1	52.73	0.09	0.77ns
	APR-A	11.22	1	11.22	5.82	0.02*
	APR-P	5939.15	1	5939.15	3.38	0.07ns
Line	AI	626.17	14	44.73	51.85	<0.001***
	RR1	171.05	14	12.22	1.70	0.08ns
	RR2	24,195	14	1728.21	1.27	0.25ns
	GPR-A	41.16	14	2.94	0.95	0.52ns
	GPR-P	10,473.03	14	748.07	1.21	0.29ns
	APR-A	65.68	14	4.70	2.43	0.01**
	APR-P	52,337.23	14	3738.37	2.13	0.02*
Treatment*Line	AI	184.54	14	13.18	15.28	<0.001***
	RR1	76.56	14	5.47	0.76	0.71ns
	RR2	17,598.18	14	1257.01	0.92	0.54ns
	GPR-A	27.21	14	1.94	0.62	0.83ns
	GPR-P	14,054.266	14	1003.876	1.63	0.09ns
	APR-A	28.207	14	2.015	1.04	0.42ns
	APR-P	14,575.254	14	1041.09	0.592	0.86ns

***: $p < 0.001$. **: $p < 0.01$. *: $p < 0.05$. ns: not significant ($p \geq 0.05$). AI: Androgenic induction; RR1: Plant regeneration per anther; RR2: Conversion efficiency (per induced structure); GPR-A: Green plant regeneration per anther; GPR-P: Green plant regeneration per total plants; APR-A: Albino plant regeneration per anther; APR-P: Albino plant regeneration per total plants.

The induction rates of embryogenic structures varied significantly among the fifteen barley genotypes, highlighting a strong genotypic effect as well as a notable influence of the cold pretreatment duration. A clear genotype × treatment interaction was observed, as the response to cold treatment differed markedly between genotypes. Genotypes showed contrasting sensitivities to cold duration. A first group, including OR 1-10 and OR 1-2, reached peak induction under T1 but suffered a significant performance decline when cold exposure was extended (Table 4). Conversely, genotypes such as OR 2-9 and OR 1-5 demonstrated a positive correlation between extended cold (T2) and embryogenic potential. In contrast, genotypes such as OR 2-9 and OR 1-5 demonstrated a significantly increased androgenic induction (AI) potential after prolonged cold pretreatment (T2), indicating that prolonged stress facilitates microspore reprogramming in these specific genetic contexts (Table 4). Under T2 conditions, OR 1-6 exhibited the highest AI, while OR 1-7 remained the least responsive. Furthermore, the relative stability observed in rihane and OR 1-6 in both treatments underscores a high embryogenic capacity, largely independent of the duration of cold exposure. These results, reinforced by the highly significant interaction between genotype and treatment (Table 3), confirm that androgenic efficacy is not universal but depends on a precisely balanced synergy between genetic background and the duration of cold pretreatment.

Table 4: Variation of androgenic indication (AI%) in embryogenic structures among fifteen barley genotypes across different cold pretreatment durations (T1 = 21 days. T2 = 35 days).

Lines	Treatment 1	Treatment 2
OR 1-2	3.66 ± 0.78^{abc}	2.91 ± 0.91^{abcde}
OR 1-10	10.43 ± 1.39^{hi}	3.37 ± 0.94^{abcd}
OR 1-8	2.76 ± 0.42^{abc}	2.10 ± 0.1^{a}
OR 1-9	2.89 ± 0.21^{abc}	3.74 ± 0.98^{abcde}
OR 2-3	2.63 ± 0.69^{abc}	3.22 ± 0.70^{abc}
rihane	2.24 ± 0.66^{a}	2.90 ± 0.19^{abc}
OR 2-13	5.17 ± 1.30^{bcde}	5.41 ± 0.48^{ef}
OR 1-5	4.29 ± 0.83^{cde}	6.78 ± 0.97^{fg}
OR 2-1	3.00 ± 1.06^{abc}	3.88 ± 0.24^{abcde}
OR 1-4	4.19 ± 0.71^{bcde}	9.62 ± 1.40^{h}
OR 1-6	11.27 ± 1.08^{i}	14.16 ± 2.17^{j}
OR 2-9	2.24 ± 0.78^{a}	7.79 ± 1.46^{g}
OR 1-7	2.37 ± 0.74^{ab}	2.09 ± 0.06^{a}
OR 1-12	2.13 ± 0.65^{a}	5.01 ± 0.37^{de}
OR 2-2	2.36 ± 0.59^{ab}	3.20 ± 1.23^{abc}

The values followed by the same letters within a column are not significantly different according to Duncan's multiple range test ($p < 0.05$).

3.3 Variation of Androgenic Regeneration Capacity

Plant regeneration efficiency, measured by overall competence (RR1) and conversion rate (RR2), was significantly influenced by the interaction between genotype and cold duration (Tables 3 and 5). Under the 21-day pretreatment (T1), a subset of genotypes demonstrated the highest regenerative capacity, notably OR 1-6 (4.25% RR1; 38.61% RR2) and OR 1-10 (3.32% RR1; 33.33% RR2), successfully converting induced structures into viable plantlets. In contrast, several lines, such as OR 1-8 and OR 1-7, remained recalcitrant to this shorter stress duration, showing no regenerative response (RR1 = 0).

Extending the cold pretreatment to 35 days (T2) generally enhanced morphogenic competence across the majority of genotypes. This improvement was most notable in OR 1-5, which achieved the highest performance of the study with an RR1 of 6.42% and an RR2 of 86.11%. Furthermore, previously unresponsive lines showed significant gains, including OR 2-13 (4.15% RR1; 72.22% RR2) and OR 1-12 (3.29% RR1; 66.67% RR2). However, a distinct group of genotypes exhibited a significant decline; for instance, OR 1-10 saw its RR1 drop to 0.61%, while rihane failed to regenerate under T2. This contrasting response suggests that while prolonged cold catalyzes the sporophytic transition in some backgrounds, it may induce inhibitory physiological stress or hormonal imbalances in others.

Table 5: Variation in plant regeneration rates (RR%) from barley anther culture (RR1: per Initial anthers; RR2: per induced structures; T1: cold pretreatment 3 weeks; T2: cold pretreatment of anthers 5 weeks).

	RR1		RR2	
Lines	Treatment 1	Treatment 2	Treatment 1	Treatment 2
OR 1-2	0.94^{ab}	1.14^{ab}	27.78^{ab}	33.33^{ab}
OR 1-10	3.32^{abc}	0.61^{ab}	33.33^{ab}	16.67^{ab}
OR 1-8	0	0	0	0
OR 1-9	0	0.81^{ab}	0	16.67^{ab}
OR 2-3	0.37^{a}	1.83^{abc}	16.67^{ab}	55.56^{ab}
rihane	0.86^{ab}	0	33.33^{ab}	0
OR 2-13	0.51^{ab}	4.15^{abc}	11.11^{a}	72.22^{ab}

Table 5: *Cont.*

	RR1		RR2	
Lines	**Treatment 1**	**Treatment 2**	**Treatment 1**	**Treatment 2**
OR 1-5	1.75^{abc}	6.42^{c}	33.33^{ab}	86.11^{b}
OR 2-1	0.81^{ab}	1.67^{abc}	33.33^{ab}	44.44^{ab}
OR 1-4	0.85^{ab}	1.99^{abc}	22.22^{ab}	20.50^{ab}
OR 1-6	4.25^{abc}	5.76^{bc}	38.61^{ab}	41.67^{ab}
OR 2-9	0	3.34^{abc}	0	41.67^{ab}
OR 1-7	0	0	0	0
OR 1-12	0	3.29^{abc}	0	66.67^{ab}
OR 2-2	0	0.71^{ab}	0	16.67^{ab}

The values followed by the same letters within a column are not significantly different according to Duncan's multiple range test ($p < 0.05$).

In summary, these results emphasize that the interaction between genotype and cold pretreatment duration critically influence barley anther culture regeneration. Optimizing barley doubled haploid production therefore requires genotype-specific to optimize both embryogenic induction and the efficient conversion of induced structures into chlorophyllous viable plantlets.

3.3.1 Regeneration of Green Viable Plants

The rate of green plant regeneration per initial anther (GPR-A) reveal significant variability among barley genotypes in their ability to produce green, viable plantlets, and this ability is influenced by the cold pretreatment duration (Table 6). Statistical analysis revealed a highly significant effect of genotype and pretreatment on the variation of this parameter (Table 3).

Under a shorter cold exposure of 21 days (T1), measurable green plant regeneration (GPR-A) was limited to a few responsive genotypes, notably OR 1-6, OR 1-10, Rihane, and OR 1-5, which exhibited low but distinct regeneration rates. The majority of other lines proved refractory to this shorter induction period, failing to produce viable green regenerants after three weeks of stress. A prolonged cold pretreatment of 35 days (T2) induced a general increase in GPR-A for several lines. Notably, OR 1-5 demonstrated the most substantial gain in green plant yield, followed by OR 1-6, OR 2-9, and OR 2-13, all showing improved performance compared to the shorter treatment.

This observation suggests that prolonged cold exposure may facilitate the stabilization of plastid differentiation or improve the survival of chlorophyllous structures in these specific genetic backgrounds. Conversely, the total loss of green plant production in OR 1-10 and rihane under T2 confirms a detrimental sensitivity to extended stress, which likely results in an irreversible physiological arrest in these sensitive genotypes (Fig. 4).

The proportion of green plants among the total number of regenerators (GPR-P) is a key indicator of the morphogenic quality of the induced structures. After a 21-day cold pretreatment (T1), the Rihane (66.67%) and OR 2-1 (33.33%) genotypes exhibited the highest frequencies of chlorophyllous plants, suggesting a superior capacity to maintain plastid integrity under moderate stress. Conversely, several lines produced exclusively albino seedlings despite successful regeneration, highlighting a basic genetic susceptibility to plastid genome degradation, even under shorter stress durations.

Extended cold pretreatment (T2) induced divergent responses regarding regeneration quality. While GPR-P significantly improved in genotypes such as OR 2-3 (50%), OR 2-9 (33.33%), and OR 1-5 (23.33%), it was completely suppressed in initially reactive lines such as OR 1-10 and Rihane. This confirms that the optimal cold pretreatment for the production of viable green plants is strictly genotype dependent. For some

genotypes, prolonged cold exposure likely exceeds the physiological threshold, shifting the developmental balance toward albinism or a complete inhibition of chlorophyll synthesis.

Figure 4: Regeneration (**a**) and acclimatization of sterile plantlets (**b**–**d**) derived from anther culture after cold pretreatment.

Table 6: Variation in chlorophyllous plant regeneration rates (GPR-A: per initial anthers; GPR-P: per total regenerated plants) among fifteen barley genotypes across different cold pretretment (T1: 21 days; T2: 35 days).

	GPR-A		GPR-P	
Lines	**Treatment 1**	**Treatment 2**	**Treatment 1**	**Treatment 2**
OR 1-2	0	0	0	0
OR 1-10	1.11^{ab}	0	22.22^{ab}	0
OR 1-8	0	0	0	0
OR 1-9	0	0	0	0
OR 2-3	0	0.88^{ab}	0	50^{ab}
rihane	0.86^{ab}	0	66.67^{b}	0
OR 2-13	0	0.98^{ab}	0	11.11^{a}
OR 1-5	0.58^{ab}	3.65^{b}	11.11^{a}	23.33^{ab}
OR 2-1	0.46^{ab}	0.63^{ab}	33.33^{ab}	16.67^{a}
OR 1-4	0	0.37^{ab}	0	16.67^{a}
OR 1-6	1.21^{ab}	2.65^{ab}	13.33^{a}	18.52^{a}
OR 2-9	0	2.56^{ab}	0	33.33^{ab}
OR 1-7	0	0	0	0
OR 1-12	0	0	0	0
OR 2-2	0	0	0	0

The values followed by the same letters within a column are not significantly different according to Duncan's multiple range test ($p < 0.05$).

Overall, these results suggest that both genotype and duration of cold pretreatment affect not only the quantity but also the quality of plant regeneration in barley anther culture. Extended cold pretreatment tends to improve the regeneration of chlorophyllous plants in some genotypes, enhancing the yield of viable green plantlets, but may reduce or inhibit it in others. This highlights the importance of optimizing cold pretreatment duration on a genotype-specific basis to maximize the production of chlorophyllous (green and viable) regenerants.

3.3.2 Regeneration of Albinos Plants

Albino plants regeneration (AR), characterized by non-photosynthetic and non-viable seedlings, is a major limiting factor for the efficiency of the studied androgenesis protocols. Analysis of variance (ANOVA) confirmed highly significant effects of genotype and cold pretreatment duration on this parameter (Table 3).

Regarding the rate of albino plant regeneration per initial anther (ARA-A), the OR 1-6 and OR 1-10 genotypes exhibited the highest rates under T1 (Table 7). While several lines, including rihane and OR 1-7, showed strong genetic resistance to albinism under T1, prolonged cold exposure (T2) led to a notable increase in albino formation in initially resistant genotypes such as OR 2-13 and OR 1-12. The proportion of albinos among the regenerators (APR-P) highlights a significant trade-off between regenerative capacity and plant viability. Under T1, OR 1-6 exhibited a predominant albinism frequency, indicating that most of its morphogenic potential resulted in non-viable plants. Prolonged stress (T2) further accentuated this imbalance in favor of albinism for OR 1-4 and OR 1-5, a phenomenon likely linked to plastid DNA deletion during microspore reprogramming. Conversely, the consistent absence of albinos in rihane and OR 1-7 in both treatments suggests a strong genetic capacity to maintain plastid integrity, even under prolonged stress conditions.

Table 7: Variation in regeneration rates of albino plants from barley anther cultures (APR-A: per initial anther; APR-P: per regenerated plant) according to different cold pretreatment durations (T1: 21 days; T2: 35 days).

	APR-A		APR-P	
Lines	**Treatment 1**	**Treatment 1**	**Treatment 1**	**Treatment 2**
OR 1-2	0.94^{abcd}	1.14^{abcd}	66.67^{ab}	66.67^{ab}
OR 1-10	2.21^{abcd}	0.61^{abcd}	44.44^{ab}	33.33^{ab}
OR 1-8	0	0	0	0
OR 1-9	0	0.81^{abcd}	0	33.33^{ab}
OR 2-3	0.34^{ab}	0.95^{abcd}	33.33^{ab}	50^{ab}
rihane	0	0	0	0
OR 2-13	0.51^{abc}	3.17^{cd}	33.33^{ab}	55.56^{ab}
OR 1-5	1.17^{abcd}	2.77^{abcd}	22.22^{ab}	76.67^{ab}
OR 2-1	0.35^{ab}	1.04^{abcd}	33.33^{ab}	50^{ab}
OR 1-4	0.85^{abcd}	1.62^{abcd}	66.67^{ab}	83.33^{ab}
OR 1-6	3.03^{bcd}	3.11^{cd}	86.67^{b}	48.15^{ab}
OR 2-9	0	0.78^{abcd}	0	33.33^{ab}
OR 1-7	0	0	0	0
OR 1-12	0	3.29^{d}	0	66.67^{ab}
OR 2-2	0	0.71^{abcd}	0	33.33^{ab}

The values followed by the same letters within a column are not significantly different according to Duncan's multiple range test ($p < 0.05$).

Based on chromosome counting using the Feulgen staining technique, all the regenerated plants were confirmed to be haploid. Following colchicine treatment, the morphological traits of the treated barley plants such as increased plant vigor, thicker stems, broader leaves, and enhanced overall growth

were consistent with successful chromosome doubling, confirming the diploid status of the previously haploid regenerants.

4 Discussion

The developmental stage of microspores remains a critical determinant of successful androgenesis, with the mid to late uninucleate stage exhibiting the highest embryogenic potential due to its cellular plasticity and ability to undergo symmetrical division [19]. Androgenesis relies on redirecting microspore development from the gametophytic to the sporophytic pathway, a process initiated by stress-induced symmetrical division that leads to callus formation and subsequent embryo development [20]. Our findings support the use of morphological markers to identify this responsive stage, offering a practical and reproducible alternative to cytological staging, provided that genotypic variability is taken into account. The genotypic differences observed in microspore development align with previous reports underscoring the significant role of genetic background in shaping androgenic responses in cereal crops [8]. Therefore, genotype-specific adjustment of spike harvesting based on reliable morphological indicators is essential for enhancing doubled haploid production in barley. Importantly, aligning morphological cues with cytological validation remains crucial for maximizing androgenesis efficiency across diverse genotypes [21].

The results of this study revealed remarkably high androgenic induction rates in spring barley, as compared to other cereals [22]. This significant interaction confirms that the signal required to trigger microspore reprogramming is strictly genotype dependent, a finding that aligns with previous reports emphasizing the decisive role of genetic background in stress perception and cellular plasticity [8,23,24]. The successful reprogramming of microspores into embryogenic structures, particularly under optimized cold pretreatment and BAC3 medium, resulted in induction frequencies that exceed those typically reported in others cereal anther culture studies [3]. It is widely acknowledged that the success of androgenesis depends on a complex interplay of factors, including donor plant genotype and physiological condition, the hormonal and nutritional composition of the induction medium, and the nature and duration of pretreatment [23]. Among these, genotype is consistently reported as the most critical determinant, as demonstrated in our study by the significant variation among the 15 barley lines. The robust embryogenic response observed in specific backgrounds confirms that despite overall genotype dependency, certain genetic combinations retain higher cellular plasticity for microspore reprogramming. These findings reaffirm that genotypic responsiveness is a limiting factor in barley androgenesis and that exploiting highly responsive lines is essential for developing efficient doubled haploid production pipelines in barley breeding programs [24]. Although several optimized protocols for inducing microspore embryogenesis have been developed, the efficiency of the process still varies significantly depending on the genotype [25].

Variation in barley regeneration rates (RR1 and RR2) across genotypes and cold pretreatment durations confirms the strong genetic control of this androgenic trait as reported by others studies [26]. Some genotypes responded well to short cold exposure, while others required longer treatment, showing genotype-specific sensitivity. The contrasting responses among genotypes suggest differences in stress perception and cellular reprogramming capacity, supporting previous reports on the strong genetic control of androgenesis in barley. High induction rates did not always lead to successful plant regeneration, emphasizing the importance of embryoid quality. Prolonged cold improved regeneration in some genotypes but inhibited it in others, likely due to stress effects. These results highlight the need to customize cold pretreatment for each genotype to enhance both induction and regeneration, crucial for efficient doubled haploid production in barley breeding. ur results suggest that the positive response of OR 1-5 and OR 2-13 genotypes to the prolonged 35-day treatment (T2) reflects a higher stress perception threshold. For

these genotypes, a shorter signal (T1) is likely insufficient to trigger the necessary metabolic changes, while the prolonged T2 signal acts as a catalyst to overcome epigenetic barriers and initiate sporophytic reprogramming. Conversely, the significant decrease observed in OR 1-10 and Rihane under T2 suggests that 35 days of cold exceeds their physiological limit. In these sensitive genotypes, excessive stress likely leads to inhibitory hormonal imbalances or irreversible developmental arrest, marking the limit of their cellular plasticity.

Albinism continues to pose a significant challenge in cereal crop androgenesis, particularly in isolated microspore or anther cultures where a high incidence of albino plant formation restricts broader application. This phenomenon is strongly influenced by genotype and is commonly observed across many monocotyledonous species such as wheat, barley, rice, triticale, oat, and rye [27]. While *in vitro* androgenesis techniques have proven effective in barley, the occurrence of albinism and its dependence on genotype remain limiting factors for the widespread production of DH plants in certain barley lines. Notably, spring barley genotypes tend to exhibit a significantly higher frequency of albino regenerants compared to winter barley [28]. Consequently, ongoing refinement of these methodologies remains a key objective within both research and breeding initiatives [24]. Although ploidy status was confirmed by chromosome counting and colchicine-induced chromosome doubling, genetic fidelity was not evaluated using molecular markers. Further molecular analyses would be useful to confirm the genetic stability of regenerated doubled haploid lines.

Albinism in androgenic cultures is a distinctive trait of cereals, with a variable occurrence rate reported across most agronomically important monocots, including barley [29]. The susceptibility to albino plant regeneration is highly genotype dependent, with frequencies ranging from as low as 1% to complete albino regeneration in certain cultivars [30]. A major factor contributing to this defect is the deletion of plastid DNA, which disrupts normal plastid development and leads to albino phenotypes. Comparative studies between green regenerants and albino regenerants in spring barley have confirmed that degradation of the plastid genome is a critical underlying cause of albinism [25].

These findings indicate that both genotype and culture conditions influence albino plant formation. High rates of albino regeneration in some genotypes can substantially limit the effective yield of viable plants, even when overall regeneration rates are favorable. Therefore, minimizing albinism through careful genotype selection and optimization of factors such as cold pretreatment duration is essential to enhance the efficiency and practicality of barley androgenesis protocols.

5 Conclusion

This study confirms that the efficiency of barley androgenesis is governed by a precisely balanced synergy between genotype and cold pretreatment duration. The highly significant genotype × treatment interactions for Androgenic Induction (AI) and plant regeneration underscore that the cellular plasticity required for microspore reprogramming is governed by genotype specific stress tolerance thresholds. While extended cold pretreatment (35 days) catalyzes morphogenic competence in the majority of lines, it triggers an irreversible physiological arrest or hormonal imbalance in sensitive genotypes like rihane, highlighting the existence of distinct biological stress thresholds. Furthermore, the complete resistance to albinism (APR) observed in Rihane and OR 1-7 suggests a robust genetic capacity to maintain plastid integrity, a trait that could be targeted for improving DH production efficiency. These findings emphasize that optimizing barley doubled haploid technology requires tailored protocols that align stress signals with the cellular plasticity of the target germplasm, thereby ensuring a reliable throughput for accelerated breeding programs.

Acknowledgement: The authors wish to thank the National Institute of Agricultural Research of Tunisia (INRAT) for hosting and supporting this research.

Funding Statement: This research was funded by the Ministry of Agriculture.

Author Contributions: The authors confirm their contribution to the paper as follows: study conception and design: Sonia Mansouri, Ali Ltifi, Leila Riahi; data collection: Sonia Mansouri, Yassmine Abidi; analysis and interpretation of results: Sonia Mansouri, Leila Riahi, Ali Ltifi; draft manuscript preparation: Sonia Mansouri, Leila Riahi. All authors reviewed and approved the final version of the manuscript.

Availability of Data and Materials: The data that support the findings of this study are available from the corresponding author, Sonia Mansouri, upon reasonable request.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

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