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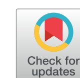
Improvement of anther cultures conditions using the Taguchi method in three cereal crops



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ABSTRACT

Background: Plant tissue cultures have the potential to reprogram the development of microspores from normal gametophytic to sporophytic pathway resulting in the formation of androgenic embryos. The efficiency of this process depends on the genotype, media composition and external conditions. However, this process frequently results in the regeneration of albino instead of green plants. Successful regeneration of green plants is affected by the concentration of copper sulfate ($CuSO_4$) and silver nitrate ($AgNO_3$) and the length of induction step. In this study, we aimed at concurrent optimization of these three factors in barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* L.), and triticale (x *Triticosecale* spp. Wittmack ex A. Camus 1927) using the Taguchi method. We evaluated uniform donor plants under varying experimental conditions of *in vitro* anther culture using the Taguchi approach, and verified the optimized conditions.
Results: Optimization of the regeneration conditions resulted in an increase in the number of green regenerants compared with the control. Statistic Taguchi method for optimization of the *in vitro* tissue culture plant regeneration *via* anther cultures allowed reduction of the number of experimental designs from 27 needed if full factorial analysis is used to 9. With the increase in the number of green regenerants, the number of spontaneous doubled haploids decreased. Moreover, in barley and triticale, the number of albino regenerants was reduced.
Conclusion: The statistic Taguchi approach could be successfully used for various factors (here components of induction media, time of incubation on induction media) at a one time, that may impact on cereals anther cultures to improve the regeneration efficiency.
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1. Introduction

Different stresses such as heat shock [1], cold treatment [2], osmotic stress [3] or anti-microtubular agents (colchicine) [4] triggered microspores develop into androgenic embryos. Regeneration of green doubled haploid (DH) plants from immature pollen requires reprogramming of microspores from the gametophytic to the sporophytic pathway. The efficiency of this process depends on the plant genotype, media composition, and external conditions used in *in vitro* trials. Fine-tuning the experimental conditions induces microspores to undergo cell division, leading to the formation of embryo-like structures, which ultimately produce plants. However, instead of green plants, albino plants are regenerated, possibly because of the inability of proplastids to form mature functional chloroplasts [5]; albino plants are unable to survive outside of the *in vitro* culture conditions.

Several factors control the switch of microspores into the sporophytic pathway; among these, media composition and environmental stresses, *i.e.*, inducing sporophytic pathway or tissue culture aging, are the easiest to control. To date, the effect of many different media components on plant regeneration has been analyzed, including plant growth regulators [6], casein hydrolysate [7], carbohydrates (for fine-tuning osmotic stress) [8,9] and micro- and macroelements [10,11,12,13,14]. However, cupric (Cu^{2+}) and silver (Ag^{+}) ions seem to be the most important. Copper plays a central role in photosynthesis, antioxidant reactions, metabolic respiration and hormone biosynthesis and perception [15,16]. However, the amount of Cu^{2+} ions in plants must be maintained under a precise control, as excess copper is toxic. In cereal crops, the benefits of copper on androgenesis rely on increasing the number of green

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regenerants (GRs), which have better survivability than albino regenerants (ARs), and the synchronization of first microspore division [17]. Other favorable effects of copper in the induction media concern general plant regeneration [10]. Copper sulfate is commonly used in plant tissue culture media, including the most frequently used Murashige and Skoog (MS) medium [18]. However, the concentration of $CuSO_4$ may need optimization for better performance. In contrast to Cu^{2+}, Ag^{+} ions do not only directly influence the number of GRs in tissue culture, but also affect *i.e.*, somatic embryogenesis, multiple shoot induction and shoot regeneration as well as rooting [19,20,21]. Silver nitrate is commonly used in plant culture media; it is soluble in water and has no toxic effects under a wide range of concentrations. The positive impact of Ag^{+} ions on the production of regenerants is derived from the ability of Ag^{+} ions to inhibit ethylene, which accumulates in culture plates during tissue culture, and influences the production and growth of regenerants [22]. Adequately adjusted concentration of silver salts in culture media significantly modulates organogenesis [23], increases the frequency of shoot regeneration [24], green plant regeneration [20] and embryo formation [25], and prevents callus necrosis, thus improving the frequency of embryogenic callus development and growth [19].

Stress conditions are necessary for the regeneration of green plants *via* androgenesis [26]. The time of tissue culture is critical for plant regeneration. The duration of *in vitro* culture and the number of subcultures [27] induces changes in plant morphology, DNA sequence and DNA methylation patterns [28]. Tissue culture over an extended period of time results in accumulation of mutations [29]. These DNA mutations/rearrangements may be manifested as deletions in chloroplast DNA (ctDNA) [30], possibly resulting in the regeneration of albino plants [31].

The greater the number of factors that need to be optimized, the larger the experimental design [32]. In a complete factorial analysis [33], optimization of three factors present at three levels requires 27 experiments. Such an approach is time consuming and expensive, and thus impractical. Using a proper statistical approach, such as the Taguchi method, may resolve this problem. The Taguchi approach is the method of choice for optimizing multiple factors [34], and relies on regression analysis and orthogonal array. Although the Taguchi method was primarily developed for technological processes, such as waste water treatment and bioremediation [35], and has been used in the healthcare industry [36], it has also proven valuable in biotechnological approaches such as fermentation for improving product yields [37], and reaction condition optimization [38]. More complex optimization problems inspired by nature require more intelligent tools called nature-inspired algorithms [39,40]. Here, the Taguchi method is suitable when the starting biological material is limited, as is the case in tissue culture experiments leading to DH production. Notably, using the statistic Taguchi method, the number of needed experiments in case of optimization three factors ($AgNO_3$, $CuSO_4$, length of induction step) and three levels of each of them, was trim down from 27 to 9. However, the use of the Taguchi method in tissue culture experiments needs to be verified.

In this study, we employed the Taguchi method for the simultaneous optimization of three factors ($CuSO_4$ and $AgNO_3$ concentrations and length of induction step) in anther cultures of barley, wheat and triticale for the regeneration of green plants.

2. Material and methods

2.1. Plant material

Spring barley cultivar NAD2 was provided by Poznan Plant Breeders LTD-Nagradowice, Poland. Winter triticale T28/2 was derived from cv. Mungis × cv. Presto cross [41], and supplied by Sylwia Oleszczuk IHAR-PIB, Poland. Winter wheat genotype Svilena was provided by Janos Pauk, Cereal Research Non-profit Limited Company, Szeged, Hungary.

2.2. Plant growth conditions

Barley, wheat and triticale plants were grown in pots (26 cm width × 23 cm height) filled with a mixture of soil and sand (soil: sand = 3:1). A total of 24 plants were used for each species, with six plants per pot. Plants were grown until the collection of spikes in a growth chamber maintained under controlled conditions (16 h light/8 h dark photoperiod, 16°C and 12°C temperature during the subjective day and night, respectively, and approximately 190 $\mu E\ m^{-2}\ s^{-1}$ light intensity using sodium lamps–light intensity was measured using the GLOptic (Poznan, Poland) spectroradiometer. During the vegetative stage, plants were fertilized (Florovit) every 2 weeks. Spikes were harvested when the microspores were in the mid to late uninucleate stage, which was determined using acetic carmine staining. Suitable spikes of barley, wheat and triticale plants were incubated in the dark at 4°C in a pot containing water for 21, 22 and 20 d, respectively. Subsequently, spikes were sterilized by soaking in 70% ethanol for 1 min, followed by soaking in 10% sodium hypochlorite for 20 min, and then washed four times with sterile distilled water [42]. Under sterile conditions, anthers were placed on species-specific semi-solid induction medium (Table 1) and incubated in the dark at 26°C until the end of callus formation. After 2–4 weeks of anther culture, androgenic embryo-like structures (1.5–3 mm in size) were transferred to the regeneration medium (Table 1).

Calli and embryo-like structures were incubated at 26°C under a 16 h light/8 h dark photoperiod (50 $\mu E\ m^{-2}\ s^{-1}$ light intensity). Green regenerated plantlets were transferred to glass flasks containing N6I rooting medium [43] supplemented with 2 mg l^{-1} indole-3-acetic acid (IAA). The developed seedlings were transferred to pots and grown in a greenhouse. The chromosome number doubled spontaneously. Species that required vernalization were incubated at 4°C under 8 h light/16 h dark photoperiod for 6 weeks (20 $\mu E\ m^{-2}\ s^{-1}$ light intensity). Spikes from randomly chosen regenerants were self-pollinated. Progenies derived from DH regenerants served as donors of explants (anthers) for regenerating plants for the optimization (Experiment 1) and verification (Experiment 2) of *in vitro* anther culture conditions.

2.3. Taguchi method

In Experiment 1, the Taguchi method was used for the simultaneous optimization of three factors ($CuSO_4$ and $AgNO_3$ concentrations and length of induction step) at three levels each, leading to a total of nine trials (M1–M9). Varying concentrations of $CuSO_4$ and $AgNO_3$ were added to the induction medium, as shown in Table 2. The length of the induction step, comprising the time from the plating of anthers on induction media to the collection of calli and subsequent transfer to

Table 1
Composition of species-specific media used for plant induction and regeneration from microspores.

Species	Induction medium[a]	Regeneration medium[b]
Barley	N6L medium containing macro- and microelements [43] supplemented with 2 mg l^{-1}, 2,4-D, 0.5 mg l^{-1} NAA and 0.5 mg l^{-1} kinetin	K4NB medium [64] supplemented with 0.225 mg l^{-1} BAP
Wheat	C17 medium [65] supplemented with 2 mg l^{-1} 2,4-D and 0.5 mg l^{-1} kinetin	190–2 medium [66] supplemented with 0.5 mg l^{-1} NAA and 1.5 mg l^{-1} kinetin
Triticale	190–2 medium [66] supplemented with 2 mg l^{-1}, 2,4-D and 0.5 mg l^{-1} kinetin	

[a] 2,4-D: 2,4-dichlorophenoxyacetic acid; NAA: napthaleneacetic acid;
[b] BAP: 6-benzylaminopurine.

Table 2
Optimisation of factors and levels of each factor for the regeneration of green plants *via* androgenesis in barley, wheat and triticale in Experiment 1 in nine trials (M1–M9) according to the Taguchi method.

Trial	Optimisation factors (Taguchi design)		
	Additional components in induction medium		Length of induction step (days)
	$CuSO_4$ (μM)	$AgNO_3$ (μM)	Barley/Wheat/Triticale
M1 (control)	0.1	0	21/35/35
M2	0.1	10	28/42/42
M3	0.1	60	35/49/49
M4	5	60	28/42/42
M5	5	0	35/49/49
M6	5	10	21/35/35
M7	10	10	35/49/49
M8	10	60	21/35/35
M9	10	0	28/42/42

regeneration media, varied with the species (Table 2). A total of nine trials (M1–M9) were performed; M1 trial served as a control, whereas trials M2–M9 represented optimization conditions. The results of all nine trials were compared by calculating the number of GRs and ARs produced per 100 anthers. Subsequently, the number of regenerants produced on regeneration media was counted, and the number of GRs and ARs per 100 anthers was calculated. These data were analyzed using the QI Macros230T software, and conditions ($CuSO_4$ and $AgNO_3$ concentrations and length of induction step) leading to the maximum production of GRs were identified. The length of induction step included time from plating anthers on induction media to the collection of calli and subsequent transfer to regeneration media. Thus, in Experiment 1, conditions optimal for green plant regeneration *via* androgenesis were determined for all three species. In Experiment 2, optimized conditions were verified by obtaining regenerants from anther cultures. The composition of regeneration media for each species was the same in both experiments.

2.4. Optimizing induction media and plant growth hormones — Experiment 1

Anthers from spikes of donor plants (24 plants per species) were plated on species-specific induction media (Table 1) containing $CuSO_4$ and $AgNO_3$ at varying concentrations; the length of induction step was carried out for different durations (Table 2). The procedure for plant regeneration was the same as that described above.

2.5. Verification of induction media — Experiment 2

In Experiment 2, conditions optimized in Experiment 1 were tested for plant regeneration from anthers obtained from 12 donor plants. The primary components of each of the induction media for the regeneration of green plants prepared as indicated in Table 1 contained extra ingredients as in Table 3. The length of induction step was adjusted as given in Table 3. A total of two trials were performed: M10 (control) and M11; trial M11 in barley, wheat and triticale has been referred to as M11B, M11W and M11T, respectively (Table 3). The number of GRs and ARs was calculated as described in Experiment 1.

Table 3
Array of factors verified in Experiment 2 according to the Taguchi method.

Trial	Optimized factors		
	Additional components in induction medium		Length of induction step (d)
	$CuSO_4$ (μM)	$AgNO_3$ (μM)	
M10 (control)	0.1	0	21 (barley), 35 (wheat and triticale)
M11B	10	30	21
M11 W	0.1	60	37
M11 T	10	0	49

2.6. Ploidy level

Ploidy of regenerants was analyzed indirectly by comparing plant morphology (plant height and leaf shape) and fertility (seed number) between donor and regenerated plants. The rate of spontaneous diploidization was calculated as follows:

$$\text{Rate of spontaneous diploidization} = \left(\frac{\text{Number of DHs}}{\text{Total number of GRs}}\right) \times 100$$

where the total number of GRs includes DH and haploid plants.

2.7. Statistical analysis

The number of GRs and ARs obtained per 100 anthers in Experiments 1 and 2 was examined for the presence of outliers using the Grubbs' tests. Outliers were removed from further analyses, and data were analyzed using analysis of variance (ANOVA), followed by Tukey's HSD test. The total number of GRs (DH and haploid) and DH regenerants in Experiments 1 and 2 were analyzed *via* linear regression. All calculations were performed using the XLSTAT 2018.1.49205 software.

3. Results

3.1. Obtaining plants

The regeneration of barley, wheat and triticale plants *via in vitro* anther culture resulted in both GRs and ARs. For each species, we evaluated 24 green, mature and fertile DHs that were phenotypically identical to the sources of explants in terms of plant height, leaf shape and fertility. The progeny of regenerants served as donor plants for the optimization (Experiment 1) as well as verification (Experiment 2) of experimental conditions for maximizing the production of GRs.

3.2. Optimizing induction media — Experiment 1

The first calli from barley, wheat and triticale anther cultures in Experiments 1 and 2 became visible after 19, 32 and 35 d, respectively. The embryo-like structures were 2–3 mm in size when transferred to regeneration media, where light conditions successfully discriminated between green and albino structures. Despite the regeneration of albino embryo-like structures, a total of 0.1–14.62 GRs per 100 anthers were obtained among all nine optimization trials (M1–M9) (Table 4). The regeneration of green plants was most difficult in barley, where the number of GRs per trial in Experiment 1 varied from 0.1 (M5) to 2.91 (M7); the corresponding number varied

Table 4
Number of green regenerants (GRs) obtained *in vitro* per 100 anthers of barley, wheat and triticale in Experiment 1.

Trial[†]	Number of GRs per 100 anthers[§]		
	Barley	Wheat	Triticale
M1 (control)	0.64^{bc}	5.52^{ab}	0.91^{b}
M2	0.67^{bc}	7.93^{ab}	0.87^{b}
M3	1.09^{bc}	14.62^{a}	1.52^{b}
M4	0.45^{bc}	14.07^{ab}	0.71^{b}
M5	0.10^{c}	5.81^{ab}	2.38^{ab}
M6	2.12^{ab}	4.33^{b}	1.17^{b}
M7	2.91^{a}	4.44^{ab}	3.79^{ab}
M8	1.77^{abc}	10.71^{ab}	4.24^{ab}
M9	0.54^{bc}	5.67^{ab}	6.06^{a}

[†] Factors analyzed in various trials (M1–M9) are described in Table 1.
[§] Different letters indicate statistically significant differences ($p < 0.05$; Tukey's HSD test).

from 4.33 (M6) to 14.62 (M3) in wheat, and from 0.71 (M4) to 6.06 (M9) in triticale. Statistical analysis of these data using ANOVA demonstrated that differences among the number of GRs obtained in Experiment 1 for all three species were significant (Table 5).

According to Tukey's grouping (Table 4), the results of experimental conditions tested among the nine trials varied for barley, wheat and triticale. In barley, M6, M7 and M8 trials grouped together and encompassed the most prospective M7 conditions. The result of M7 trial was several-fold better than that of the M5 trial, which was different from the M6–M8 group. In wheat and triticale, M3 and M9 trials were the most promising (Table 4). However, in wheat the M3 trial was a member of a larger group of trials with the M6 being distinct from the M3 one. In triticale the M9 trial was in the group of encompassing M5, M7 and M8 experimental conditions. In wheat, the number of GRs in M3 trial was 3-fold higher than that in M6. In triticale, the M9 trial produced 8-fold more GRs than the weakest trial (M4). In barley and triticale, the best conditions produced significantly greater number of GRs than control conditions (M1).

3.3. Verification of induction media — Experiment 2

In barley, the Taguchi approach suggested slightly distinct conditions in Experiment 2 than those used in Experiment 1. This difference was because of the addition of $AgNO_3$ (30 μM) and induction for a minimum time of 21 d (Table 3). In wheat and triticale, concentrations of $CuSO_4$ and $AgNO_3$ used were the same as those that produced the highest number of GRs in Experiment 1; however, the length of induction decreased in wheat and increased in triticale, compared with the length of induction in Experiment 1 (Table 3). For all three species, Experiment 2 resulted in a different number of GRs (0.55–7.99) and ARs (0.92–9.10) compared with Experiment 1 (Table 6). In Experiment 2, the lowest and highest numbers of GRs were obtained from triticale [0.55 (M10) and 1.61 (M11)] and wheat [4.95 (M10) and 7.99 (M11)], respectively (Table 6). The number of GRs produced in M11 trial was at least 2-fold higher than that in the M10 (control) for all three species. In parallel to the increase in the number of GRs in Experiment 2, the number of ARs also increased. However, this increase was higher in barley and wheat, and remained at the same level as in triticale compared M10 to M11 in every species.

Despite the differences in numbers of GRs and ARs in Experiment 2, results of ANOVA showed that the model was significant only for the number of GRs produced from triticale (Table 7), and non-significant for ARs from all three species in Experiment 2 (Table 7).

According to the results of Tukey's HSD test, the number of GRs in wheat and barley showed no significant differences between M10 (control) and M11 trials, although this number was 2-fold higher in M11 trial compared with M10 (Table 6). In triticale, the number of GRs in M11 trial was 3-fold higher than that in M10 (Table 6). A similar analysis of ARs failed to identify any significant differences between M10 and M11 trials in all three species. Nonetheless, the number of ARs in M11 trial was increased by *ca.* 30% in barley and 40% in wheat compared with M10, although no difference was observed in triticale. Within the M11 trial, the number of ARs was 4-fold higher in barley and *ca.* 2-fold lower in wheat and triticale than the number of GRs. Moreover, analysis of GR/AR ratio under control (M10) and M11 conditions in Experiment 2 showed that the number of ARs in barley and triticale decreased in M11; however, there was no evident gain in wheat (Table 8).

Table 5
Analysis of the number of green regenerants (GRs) obtained from anther culture of barley, wheat and triticale genotypes in Experiment 1 using ANOVA.

Species	*F*-value	*p*-Value
Barley	5.415	0.0001
Wheat	2.921	0.005
Triticale	3.193	0.002

Table 6
Number of green regenerants (GRs) and albino regenerants (ARs) obtained *in vitro* per 100 anthers of barley, wheat, and triticale in Experiment 2 according to the Taguchi method.

Plants	Trial[†]	Species[§]		
		Barley	Wheat	Triticale
GRs	M10	0.95[a]	4.95[a]	0.55[b]
	M11	1.97[a]	7.99[a]	1.61[a]
ARs	M10	6.45[a]	2.21[a]	1.05[a]
	M11	9.10[a]	3.67[a]	0.92[a]

[†] Factors analyzed in M10 (control) and M11 trials are described in Table 3.
[§] Different letters indicate statistically significant differences ($p < 0.05$; Tukey's HSD test).

3.4. Haploids and DHs derived in optimization and verification experiments

Some GRs obtained in Experiments 1 and 2 varied in morphology compared with donor plants. Such regenerants appeared to be haploids. The DH regenerants obtained in Experiments 1 and 2 were identical to donor plants at the morphological level. Based on the number of GRs that appeared fertile, spontaneous duplication varied from 0% (triticale; M3, M6 and M8) to 89% (barley; M5) in Experiment 1, and from 4.65% (wheat; M11) to 50% (barley; M11) in Experiment 2 (Table 9).

Linear regression analysis of the total number of GRs (DH and haploid) and DH regenerants revealed a correlation (r) of -0.525; however, this model was not significant ($F = 2.176$, $p < 0.152$). The coefficient of determination (R^2) was 0.28, suggesting that 28% of the variability among all GRs explained the data for DHs.

4. Discussion

DH plants are a useful tool in plant breeding [44]; these serve as genetically uniform homozygous lines for genetic analysis and modern breeding programs [45,46]. Despite the evident utility of DH lines, many aspects of DH production need improvement. For example, the efficiency of production of GRs needs to be increased in many plant species including cereals such as wheat [47,48], and the number of ARs needs to be reduced [47]. Additionally, the problem of spontaneous diploidization in DHs needs to be resolved [49]. Adjusting the concentrations of chemicals in the induction and regeneration media and fine-tuning the length of induction step have been used for modifying the efficiency of green plant regeneration [50,51]. However, it is highly challenging to test multiple factors simultaneously in one experiment, as this requires several trials. From the experimental point of view, availability of morphologically and genetically uniform and stable regenerants as the starting material is of fundamental importance so that the resulting progeny can serve as a donor of explants.

In this study, care was taken to assure that the starting plant material was highly uniform, at least at the morphological level. Thus, donor plants originated from DH plants. Previous investigations suggest that

Table 7
Analysis of the number of green regenerants (GRs) and albino regenerants (ARs) obtained from anther culture of barley, wheat and triticale genotypes in Experiment 2 using ANOVA.

Plants	Species	*F*-value	*p*-Value
GRs	Barley	2.256	0.142
	Wheat	0.814	0.375
	Triticale	12.716	0.001
ARs	Barley	1.357	0.251
	Wheat	0.455	0.505
	Triticale	0.226	0.636

Table 8
Ratio of green regenerants (GRs) to albino regenerants (ARs) produced *via* anther culture of barley, wheat and triticale genotypes under control (M10) and verified conditions (M11) in Experiment 2.

Experimental conditions	GR:AR		
	Barley	Wheat	Triticale
M10	1:6	1:0.5	1:2
M11	1:4	1:0.5	1:0.5

in some cereals, such as barley, the generative progeny of DH plants is a better choice as a donor of explants, as it is stable both at the genetic and epigenetic levels [45]. Unfortunately, this is not always the case, as evidenced in triticale [52]. Nonetheless, morphological differences among the generative progeny of barley and triticale are not observed [46,53]. This is consistent with the current study as a single cycle of self-pollination would suffice for the production of sufficiently uniform DHs progeny from an individual genotype of barley, wheat and triticale for further experiments dedicated towards increasing the recovery of GRs *via* anther culture. However, optimization of any *in vitro* tissue culture technique requires several tests. Generally, if several factors need to be optimized, an orthogonal array is used [54]. Here, we decided to employ the Taguchi method, which relies on mathematical regression [34]. The Taguchi method substantially reduced the number of trials needed, diminishing the total number of donor plants required. Thus, instead of 27 trials, only nine trials (including a control) were needed for the simultaneous optimization of $CuSO_4$ and $AgNO_3$ concentrations in the induction medium and the length of induction step.

Optimization of conditions in Experiment 1 demonstrated that all tested factors ($CuSO_4$ and $AgNO_3$ concentrations and the length of induction step) were essential for the regeneration of green plants. Moreover, each of the three species tested (barley, wheat and triticale) exhibited different requirements for different levels of each factor used in Experiment 1.

Among the nine trials performed, conditions used in M7 trial appeared to be the most promising for the production of GRs in barley, while those used in the M5 trial were the worst. In the M7 trial, 10 μM each of $CuSO_4$ and $AgNO_3$ was added to the induction medium, whereas in the M5 trial, which produced the lowest number of GRs, only 5 μM $CuSO_4$ was added to the induction medium (without $AgNO_3$); the length of induction step was the same in M5 and M7 trials. Comparison of the number of GRs suggests the beneficial effect of $CuSO_4$ and adverse influence of $AgNO_3$ on green plant regeneration. The effect of the addition of $CuSO_4$ (10–80 μM) to liquid pre-treatment medium or culture medium on green plant regeneration from barley anther culture has been examined previously [12]. Although the number of GRs in our study (0.1–2.91) was lower than that in the study of Jacquard et al. [12] (0–106.6), the increase in the number of regenerated plants was observed. It should be stressed that the concentration of $CuSO_4$ in culture medium cannot be increased endlessly; at concentrations >80 μM, microspore embryogenesis is completely inhibited [12]. Additionally, Makowska et al. [10] reported an increase in the number of GRs in barley *via* isolated microspore culture in the presence of 10 μM $CuSO_4$. These data are congruent with the results of Experiment 1 in this study, suggesting that 10 μM $CuSO_4$ is ideal for maximizing the production of GRs. Nevertheless, there are discrepancies in the number of GRs between our results and those of others. We obtained fewer GRs (0.1–2.91) than Jacquard and co-workers (0–106.6) [12]. This incongruity could be due to the difference in genotypes used, as the genotype affects the ability of androgenesis [55]. Additionally, differences could have originated from poor reproducibility under *in vitro* culture conditions. This possibility is supported by the presence of outliers in our experiments that were removed from data analysis to avoid misinterpretations. The highest number of GRs was obtained in the presence of 10 μM each of $CuSO_4$ and $AgNO_3$. Thus, the highest supplementation of $AgNO_3$ (60 μM) alone did not guarantee the best production of GRs (M3). Notably, none of the $AgNO_3$ concentrations (0–60 μM; M1, M2 and M3) in the presence of lowest $CuSO_4$ concentration led to an increase in the number of GRs. In barley, the highest number of GRs was obtained at the maximum concentration of $CuSO_4$ (10 μM) and moderate concentration of $AgNO_3$ (10 μM) examined. Interestingly, the barley genotype used in this study seems to prefer an average length of induction compared with the control. Although the reason for this observation is not apparent, we speculate that very short duration of induction is not sufficient for the regeneration of a high number of green plants, whereas prolonged induction may be toxic to barley anther cultures or may make the medium toxic.

In wheat, M3 and M4 trials produced a greater number of GRs than M6 and M7 trials, suggesting that wheat prefers the lowest $CuSO_4$ (0.1 μM) concentration and the highest $AgNO_3$ (60 μM) concentration, in conjunction with extended length of induction step. If the concentrations of $CuSO_4$ were increased and $AgNO_3$ were decreased, the quantity of regenerants was reduced. Similarly the higher concentration of $AgNO_3$ (15–30 μM) in tested variant than in control increased the frequency of embryoid formation in three out of four genotypes studied [25]. In light of the known effects of Cu^{2+} ions, it is unclear why wheat prefers low rather than high $CuSO_4$ concentration in the induction medium. It is possible that anther cultures of wheat, or specifically of the winter wheat genotype Svilena, are more sensitive to Cu^{2+} ion toxicity. Different reactions of other wheat genotypes towards the presence of Cu^{2+} ions have been observed in *in vitro* cultures, supporting the latter hypothesis [56,57]. The androgenic response of wheat genotypes to $CuSO_4$ appears to be broad (0.1–100 μM). Purnhauser and Gyulai [58] reported that in the presence of 100 μM $CuSO_4$, the mean shoot number per regenerating callus was the same as that in the presence of 10 μM $CuSO_4$. In wheat regeneration in Experiment 1, intermediate (and extended) length of induction step produced better results than that used in the control trial (M1), similar to that in barley. Thus, extended length of induction step strengthens the action of components in the induction medium.

In triticale, conditions of the M9 trial favored the production of GRs, whereas those of the M4 trial did not. Thus, the highest concentration of $CuSO_4$ and absence of $AgNO_3$ in anther culture induction medium promotes the production of GRs from triticale, whereas intermediate $CuSO_4$ and high $AgNO_3$ concentrations reduce the number of GRs. The length of induction step seemed to have no significant effect on the production of GRs. However, Purnhauser and Gyulai [58] reported that concentrations of $CuSO_4$ (0–100 μM) and $AgNO_3$ (1–100 μM) affect the number of regenerated shoots and roots in triticale. The authors showed that $CuSO_4$ enhances shoot regeneration, with the highest regeneration within the 0.1–1 μM range. At a concentration of 10 μM

Table 9
Percentage of spontaneous diploidization among green regenerants (GRs) derived from anther culture of barley, wheat and triticale genotypes in Experiments 1 and 2.

	Trial[a]	Spontaneous diploidization (%)		
		Barley	Wheat	Triticale
Experiment 1	M1 (control)	56.82	12.82	5.88
	M2	64.29	16.67	27.27
	M3	42.86	2.94	0.00
	M4	50.00	3.03	33.33
	M5	88.89	6.45	44.00
	M6	53.33	2.00	0.00
	M7	45.45	15.15	20.00
	M8	55.00	25.71	0.00
	M9	85.71	16.67	10.00
Experiment 2	M10 (control)	56.82	11.39	64.29
	M11	50	4.65	40

[a] Factors analyzed in various trials performed in experiment 1 (M1–M9) and experiment 2 (M10–M11) are described in Tables 2 and 3, respectively.

$CuSO_4$, the average number of shoots regenerated per callus decreased. Further increase in $CuSO_4$ concentration (up to 100 μM) resulted in a further decrease in the number of regenerated shoots. These results on triticale shoot regeneration are consistent with the production of green plants from triticale anther culture. Interestingly, a wide range of $AgNO_3$ concentrations (1–100 μM) has been shown to improve shoot regeneration in triticale compared with the control lacking Ag^+ ions and containing Cu^{2+} ions at a low concentration [58]. However, better shoot producing calli are observed in triticale at 1–10 μM $AgNO_3$ than at 100 μM $AgNO_3$. In our experiments, the presence of $AgNO_3$ reduced the number of green plants, when the concentration of $CuSO_4$ was 0.1–5 μM. By contrast, $AgNO_3$ slightly affected green plant regeneration if the concentration of $CuSO_4$ was high (M7, M8 trials). These results suggest that at least at high $CuSO_4$ concentrations, $AgNO_3$ has limited effects on green plant and shoot regeneration in triticale. The effect of the length of induction step on green plant regeneration from triticale anther culture was similar to that in other crop species.

Our results suggest that triticale and barley have similar requirements for $CuSO_4$ but different preferences for $AgNO_3$ in the induction media. The requirement of wheat for $CuSO_4$ and $AgNO_3$ was different from that of barley and triticale. We suggest that the distinct preferences of the three species for $CuSO_4$ and $AgNO_3$ concentrations stem from different mechanisms of induction of plant regeneration in *in vitro* anther cultures among these species. However, these mechanisms remain unclear and need further investigation. Moreover, it is not clear why, in all three species, longer induction resulted in a higher number of GRs than shorter induction. While it was not the goal of the present study, it would be of value to better understand the action of the studied factors on biochemical processes involved in green plant regeneration in the three species.

The Taguchi approach allowed the identification of optimized conditions necessary for increasing the production of GRs. However, the number of GRs obtained in Experiment 2 was suboptimal compared with that those in Experiment 1 for all three species. This difference may be due to uncontrollable factors that affect *in vitro* tissue culture [59,60]. To avoid this problem, biological replicates are performed. However, because of the shortage of donor plants and for reducing the experiment size, we could not perform biological replicates. Instead, we removed outliers and used statistics to predict *in vitro* culture conditions optimal for the generation of GRs.

Using the Taguchi method, the same induction media composition was identified as optimum for producing GRs from wheat and triticale in Experiment 1, although there were differences in the length of induction step between the two species. The verification conditions used for barley in Experiment 2 indicated that higher $AgNO_3$ concentration and shorter induction than that determined in Experiment 1 resulted in a suboptimal number of GRs. Such outcomes are not expected using the Taguchi approach. This cannot be attributed to poor reproducibility of *in vitro* culture trials, as we excluded outliers from data analysis. Moreover, control conditions in the Experiments 1 and 2 used for barley, wheat and triticale were identical. Notably, most of the changes in investigated factors suggested by the Taguchi approach affected the length of induction step in all three species and resulted in a suboptimal number of green plants, compared with the optimisation conditions in Experiment 1. Thus, either the method failed to optimize time, or we did not choose the correct length of induction step in Experiment 1. Alternatively, the length of induction step might be the most problematic issue itself, and needs further investigation. Nonetheless, in all three species, verification conditions resulted in 2–3-fold higher number of GRs than the respective control conditions (Table 6). Others have also reported a difference in the number of GRs between optimized and control conditions, although this difference is not always significant [10]. This is consistent with our data obtained in M10 and M11 trials (Experiment 2) for barley and wheat but not for triticale, as indicated by ANOVA (Table 8). Therefore, removing outliers from data analyses may not lead to the evaluation of the best results during verification using the Taguchi method. Nevertheless, this approach proved to be useful and should be the method of choice in multifactorial optimization experiments, with limited availability of experimental material.

Optimization of conditions for maximizing the production of GRs reduced the number of ARs in barley and triticale, but not in wheat. The obtained data, at least in barley and triticale, could be explained by the action of $CuSO_4$. A positive effect on the production on GRs *vs* ARs was observed in barley when added $CuSO_4$ to the regeneration media [61] and to pre-treatment solutions [17]. Such supplementation seems to be a general rule when regeneration is performed *via* androgenesis [58]. Additionally, in wheat, $CuSO_4$ has been reported to increase the number of GRs and reduce the number of ARs [62], but this was not observed in our experiments. Since the only difference between control trial (M10) and other trials in Experiment 2 for wheat was related to the length of induction, we speculate that prolonged induction does not affect the GR/AR ratio. The lack of evident reduction in the number of ARs may be due to poor reproducibility of *in vitro* tissue cultures or the lack of a sufficient number of biological repeats.

In all three species, spontaneous diploidization observed in Experiment 2 was lower than that observed in the respective controls (M10) (Table 9). In Experiment 1, the best results for barley were obtained at low $CuSO_4$ concentration, no $AgNO_3$ and 35 days of induction step. However, the Taguchi approach suggested that increasing the concentrations of both $CuSO_4$ and $AgNO_3$ (Table 3) and shortening the induction period to 21 days would lead to suboptimal results. Thus, modifications suggested by the method negatively influenced spontaneous diploidization. The same was true in wheat, where optimization conditions were comparable with the best verification conditions for media composition but were different for the length of induction and resulted in a low rate of spontaneous diploidization (Table 9). In triticale, spontaneous diploidization was lower in Experiment 2 compared with Experiment 1, although $AgNO_3$ and $CuSO_4$ concentrations in the induction medium in both experiments were the same. The difference between the experiments for triticale reflected the prolonged induction step in Experiment 2. Thus, in our study, conditions for reducing spontaneous diploidization were incongruent with those for increasing the production of GRs. This is consistent with the study of Mirzaei et al. [63] in wheat; the authors reported a negative relationship between embryogenesis and spontaneous diploidization. It should be stressed, however, that our aim was not to increase the number of DHs but to generate more green plants, as spontaneous genome duplication is not a problem in barley and triticale.

An encouraging highlight of our study was the relationship between the total number of GRs (DH and haploid) and DHs. Based on optimized conditions and linear regression data, we noted that the total number of GRs contrasted the number of spontaneously originated DHs. Our results suggest that this phenomenon is unique to cereals, which to the best of our knowledge has not been reported previously. Possibly, the presence of $CuSO_4$ or $AgNO_3$ influences spontaneous chromosome doubling. Regardless, an increase in the number of GRs is preferred, as DH plants may be easily generated using antimitotic agents; the greater the number of GRs, the higher the number of potential DHs.

5. Conclusion

Optimization of *in vitro* anther cultures of barley, wheat and triticale using the Taguchi approach enabled the identification of regeneration conditions that favor an increase in the number of GRs compared with control conditions. The Taguchi method reduced the number of experiments needed from 27 to 9, even though three factors were tested at three levels each. Since our goal was to obtain green plants

from *in vitro* anther culture, we tested the effect of $CuSO_4$ and $AgNO_3$ concentrations and the length of induction. All three species showed distinct responses towards the concentration of salts in the induction medium and length of induction step. The optimized regeneration conditions showed that with an increase in the number of GRs, the number of DHs decreased. Moreover, under optimized conditions, the GR/AR ratio decrease in barley and triticale. Optimized conditions appeared to be the best compared with those used for the optimization; this difference could be the result of changes in the length of induction, as it was the most discriminating factor between controls and optimized conditions. Alternatively, differences could be attributed to unknown factors in tissue culture. Overall, we conclude that the Taguchi approach could be successfully used for the simultaneous optimisation of multiple factors affecting *in vitro* anther culture.

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Declaration of competing interest

The authors declare no competing interests.

References

[1] Dubas E, Custers J, Kieft H, et al. Microtubule configurations and nuclear DNA synthesis during initiation of suspensor-bearing embryos from *Brassica napus* cv. Topas microspores. Plant Cell Rep 2011;30(11):2105–16. https://doi.org/10.1007/s00299-011-1117-8. 21779827.

[2] Tenhola-Roininen T, Tanhuanpää P, Immonen S. The effect of cold and heat treatments on the anther culture response of diverse rye genotypes. Euphytica 2005; 145(1–2):1–9. https://doi.org/10.1007/s10681-005-8229-6.

[3] Bal U, Elliatioglu S, Abak K. Induction of symmetrical nucleus division and multi-nucleate structures in microspores of eggplant (*Solanum melongena* L.) cultured *in vitro*. Sci Agric 2009;66(4):535–9. https://doi.org/10.1590/S0103-90162009000400016.

[4] Li JR, Zhuang FY, Qu CG, et al. Microspore embryogenesis and production of haploid and doubled haploid plants in carrot (*Daucus carota* L.). Plant Cell Tiss Org Cult 2012; 112(3):275–87. https://doi.org/10.1007/s11240-012-0235-5.

[5] Makowska K, Oleszczuk S. Albinism in barley androgenesis. Plant Cell Rep 2014;33 (3):385–92. https://doi.org/10.1007/s00299-013-1543-x. 24326697.

[6] Esteves P, Clermont I, Marchand S, et al. Improving the efficiency of isolated microspore culture in six-row spring barley: II—Exploring novel growth regulators to maximize embryogenesis and reduce albinism. Plant Cell Rep 2014;33(6):871–9. https://doi.org/10.1007/s00299-014-1563-1. 24519013.

[7] Sriskandarajah S, Sameri M, Lerceteau-Köhler E, et al. Increased recovery of green doubled haploid plants from barley anther culture. Crop Sci 2015;55(6):2806. https://doi.org/10.2135/cropsci2015.04.0245.

[8] Wojnarowiez G, Caredda S, Devaux P, et al. Barley anther culture: assessment of carbohydrate effects on embryo yield, green plant production and differential plastid development in relation with albinism. J Plant Physiol 2004;161:747–55. https://doi.org/10.1078/0176-1617-01061. 15266723.

[9] Slama-Ayed O, De Buyser J, Picard E, et al. Effect of pre-treatment on isolated microspores culture ability in durum wheat (*Triticum turgidum* subsp. durum Desf.). J Plant Breed Crop Sci 2010;2(2):30–8.

[10] Makowska K, Oleszczuk S, Zimny J. The effect of copper on plant regeneration in barley microspore culture. Czech J Genet Plant Breed 2017;53(1):17–22. https://doi.org/10.17221/82/2016-cjgpb.

[11] Abdollahi MR, Shirin R. Production and conversion of haploid embryos in chickpea (*Cicer arietinum* L.) anther cultures using high 2,4-D and silver nitrate containing media. Plant Cell Tiss Organ Cult 2018;133(1):39–49. https://doi.org/10.1007/s11240-017-1359-4.

[12] Jacquard C, Nolin F, Hecart C, et al. Microspore embryogenesis and programmed cell death in barley: Effects of copper on albinism in recalcitrant cultivars. Plant Cell Rep 2009;28(9):1329–39. https://doi.org/10.1007/s00299-009-0733-z. 19529940.

[13] Echavarri B, Soriano M, Cistué L, et al. Zinc sulphate improved microspore embryogenesis in barley. Plant Cell Tiss Organ Cult 2008;93(3):295–301. https://doi.org/10.1007/s11240-008-9376-y.

[14] Germanà MA, Crescimanno FG, Motisi A. Factors affecting androgenesis in *Citrus clementina* Hort. ex Tan. Advances in Horticultural Science 2000;14(2):43–51.

[15] Penarrubia L, Romero P, Carrio-Segui A, et al. Temporal aspects of copper homeostasis and its crosstalk with hormones. Front Plant Sci 2015;6:255. https://doi.org/10.3389/fpls.2015.00255. 25941529.

[16] Festa RA, Thiele DJ. Copper: An essential metal in biology. Curr Biol 2011;8(21): 877–83. https://doi.org/10.1016/j.cub.2011.09.040. 22075424.

[17] Wojnarowiez G, Jacquard C, Devaux P, et al. Influence of copper sulfate on anther culture in barley (*Hordeum vulgare* L.). Plant Sci 2002;162:843–7. https://doi.org/10.1016/S0168-9452(02)00036-5.

[18] Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue culture. Physiol Plantarum 1962;15:473–97. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x.

[19] Wu LM, Wei YM, Zheng YL. Effects of silver nitrate on the tissue culture of immature wheat embryos. Russ J Plant Physiol 2006;53(4):530–4. https://doi.org/10.1134/S1021443706040157.

[20] Jha AK, Dahleen LS, Suttle JC. Ethylene influences green plant regeneration from barley callus. Plant Cell Rep 2007;26:285–90. https://doi.org/10.1007/s00299-006-0252-0. 17043878.

[21] Würschum T, Tucker MR, Maurer HP, et al. Ethylene inhibitors improve efficiency of microspore embryogenesis in hexaploid triticale. Plant Cell Tiss Organ Cult 2015;122 (3):751–7. https://doi.org/10.1007/s11240-015-0808-1.

[22] Tiainen T. Influence of ethylene in microspore embryogenesis. In: Jain SM, Sopory SK, Veilleux RE, editors. In vitro haploid production in higher plants. Current plant science and biotechnology in agricultureDordrecht: Springer; 1996. p. 177–87. https://doi.org/10.1007/978-94-017-1860-8_10.

[23] Paladi RK, Rai AN, Penna S. Silver nitrate modulates organogenesis in *Brassica juncea* (L.) through differential antioxidant defense and hormonal gene expression. Sci Hortic 2017;226:261–7. https://doi.org/10.1016/j.scienta.2017.08.038.

[24] Purnhauser L, Medgyesy P, Czakó M, et al. Stimulation of shoot regeneration in *Triticum aestivum* and *Nicotiana plumbaginifolia* Viv. tissue cultures using the ethylene inhibitor $AgNO_3$. Plant Cell Rep 1987;6(1):1–4. https://doi.org/10.1007/bf00269725. 24248436.

[25] Ghaemi M, Sarrafi A, Alibert G. The effects of silver nitrate, colchicine, cupric sulfate and genotype on the production of embryoids from anthers of tetraploid wheat (*Triticum turgidum*). Plant Cell Tiss Organ Cult 1994;36:355–9. https://doi.org/10.1007/BF00046093.

[26] Islam SM, Tuteja N. Enhancement of androgenesis by abiotic stress and other pretreatments in major crop species. Plant Sci 2012;182:134–44. https://doi.org/10.1016/j.plantsci.2011.10.001. 22118624.

[27] Ładyżyński M, Burza W, Malepszy S. Relationship between somaclonal variation and type of culture in cucumber. Euphytica 2002;125:349–56. https://doi.org/10.1023/A:1016017825907.

[28] Rival A, Ilbert P, Labeyrie A, et al. Variations in genomic DNA methylation during the long-term in vitro proliferation of oil palm embryogenic suspension cultures. Plant Cell Rep 2013;32:359–68. https://doi.org/10.1007/s00299-012-1369-y. 23179461.

[29] López CMR, Bravo HS, Wetten AC, et al. Detection of somaclonal variation during cocoa somatic embryogenesis characterised using cleaved amplified polymorphic sequence and the new freeware Artbio. Mol Breeding 2010;25(3):501–16. https://doi.org/10.1007/s11032-009-9348-x.

[30] Abe T, Li N, Togashi A, et al. Large deletions in chloroplast DNA of rice calli after long-term culture. J Plant Physiol 2002;159:917–23. https://doi.org/10.1078/0176-1617-00815.

[31] Mozgova GV, Zaitseva OI, Lemesh V. Structural changes in chloroplast genome accompanying albinism in anther culture of wheat and triticale. Cereal Res Commun 2012;40(4):467–75. https://doi.org/10.1556/CRC.40.2012.0007.

[32] Rao RS, Kumar CG, Prakasham RS, et al. The Taguchi methodology as a statistical tool for biotechnological applications. Biotechnol J 2008;3:510–23. https://doi.org/10.1002/biot.200700201. 18320563.

[33] Collins LM, Dziak JJ, Li R. Design of experiments with multiple independent variables: A resource management perspective on complete and reduced factorial designs. Psychological methods 2009;14(3):202–24. (https://doi.org/10.1037/a0015826 PMid: 19719358).

[34] Taguchi G. In: U., editor. Introduction to quality engineering. White Plains: NY: Asian Productivity Organization; 1986. p. 191.

[35] Daneshvar N, Khataee AR, Rasoulifard MH, et al. Biodegradation of dye solution containing malachite green: optimization of effective parameters using Taguchi method. J Hazard Mater 2007;143:214–9. https://doi.org/10.1016/j.jhazmat.2006.09.016. 17052836.

[36] Tanfous NGB, Kallel H, Jarboui MA, et al. Expression of *Pichia pastoris* of a recombinant scFv form of MAb 107, an anti human CD11b integrin antibody. Enzyme Microb Technol 2006;38:636–42. https://doi.org/10.1016/j.enzmictec.2005.07.014.

[37] Sirisansaneeyakul S, Luangpipat T, Vanichsriratana W, et al. Optimization of lactic acid production by immobilized *Lactococcus lactis* IO-1. J Ind Microbiol Biotechnol 2007;34:381–91. https://doi.org/10.1007/s10295-007-0208-6. 17318489.

[38] Ramakrishna U, Kingston JJ, Sripathi MH, et al. Taguchi optimization of duplex PCR for simultaneous identification of *Staphylococcus aureus* and *Clostridium perfringens* alpha toxins. FEMS Microbiol Lett 2013;340(2):93–100. https://doi.org/10.1111/1574-6968.12070. 23278425.

[39] Wang GG, Deb S, Dos Santos Coelho L. Earthworm optimisation algorithm: A bio-inspired metaheuristic algorithm for global optimisation problems. Int J Bio-Inspir Com 2018;12(1):1–22. https://doi.org/10.1504/IJBIC.2015.10004283.

[40] Wang GG, Deb S, Gao X, et al. A new metaheuristic optimisation algorithm motivated by elephant herding behaviour. Int J Bio-Inspir Com 2016;8(6):394–409. https://doi.org/10.1504/IJBIC.2016.081335.

[41] Tyrka M, Oleszczuk S, Rabiza-Swider J, et al. Populations of doubled haploids for genetic mapping in hexaploid winter triticale. Mol Breeding: new strategies in plant improvement 2018;38(4):46. https://doi.org/10.1007/s11032-018-0804-3.. PMid:29623004.

[42] Oleszczuk S, Sowa S, Zimny J. Direct embryogenesis and green plant regeneration from isolated microspores of hexaploid triticale (*Triticosecale* Wittmack) cv. Bogo. Plant Cell Rep 2004;22:885–93. https://doi.org/10.1007/s00299-004-0796-9.

[43] Chu CC. The N6 medium and its applications to anther culture of cereal crops. Proc. Symp. Plant Tissue Culture 25–30 May 1978. Pekin: Science Press; 1978. p. 43–50.

[44] Germanà MA. Anther culture for haploid and doubled haploid production. Plant Cell Tiss Organ Cult 2010;104(3):283–300. https://doi.org/10.1007/s11240-010-9852-z.
[45] Bednarek PT, Orłowska R, Koebner RMD, et al. Quantification of the tissue-culture induced variation in barley (*Hordeum vulgare* L.). BMC Plant Biology 2007;7(10). (https://doi.org/10.1186/1471-2229-7-10 PMid: 17335560).
[46] Machczyńska J, Zimny J, Bednarek P. Tissue culture-induced genetic and epigenetic variation in triticale (× *Triticosecale* spp. Wittmack ex A. Camus 1927) regenerants. Plant Mol Biol 2015;89(3):279–92. https://doi.org/10.1007/s11103-015-0368-0. 26337939.
[47] Lantos C, Pauk J. Anther culture as an effective tool in winter wheat (*Triticum aestivum* L.) breeding. Russ J Genet 2016;52(8):794–801. https://doi.org/10.1134/s102279541608007x.
[48] Bokore FE, Knox RE, Cuthbert RD, et al. Effects of media supplements on doubled haploid production in durum wheat. Can J Plant Sci 2016;97(1):65–71. https://doi.org/10.1139/cjps-2016-0066.
[49] Castillo AM, Cistué L, Vallés MP, et al. Chromosome doubling in monocots. In: Touraev A, Forster BP, Jain SM, editors. Advances in haploid production in higher plants. Dordrecht: Springer; 2009. p. 329–38. https://doi.org/10.1007/978-1-4020-8854-4_27.
[50] Puolimatka M, Pauk J. Effect of induction duration and medium composition on plant regeneration in wheat (Triticum aestivum L.). J Plant Physiol 2000;156:197–203. https://doi.org/10.1016/S0176-1617(00)80306-5.
[51] Chen QF, Wang CL, Lu YM, et al. Anther culture in connection with induced mutations for rice improvement. Euphytica 2001;120(3):401–8. https://doi.org/10.1023/a:1017518702176.
[52] Machczyńska J, Orłowska R, Mańkowski DR, et al. DNA methylation changes in triticale due to *in vitro* culture plant regeneration and consecutive reproduction. Plant Cell Tiss Organ Cult 2014;119(2):289–99. https://doi.org/10.1007/s11240-014-0533-1.
[53] Orłowska R, Machczyńska J, Oleszczuk S, et al. DNA methylation changes and TE activity induced in tissue cultures of barley (*Hordeum vulgare* L.). J Biol Res-Thessalon 2016;23:19. https://doi.org/10.1186/s40709-016-0056-5.. PMid: 27508170.
[54] EL-Moslamy SH, Elkady MF, Rezk HA, et al. Applying Taguchi design and large-scale strategy for mycosynthesis of nano-silver from endophytic *Trichoderma harzianum* SYA.F4 and its application against phytopathogens. Sci Rep-UK 2017;7:45297. https://doi.org/10.1038/srep4529.. PMid: 28349997.
[55] Devaux P, Kasha KJ. Overview of barley doubled haploid production. In: Touraev A, Forster BP, Jain SM, editors. Advances in haploid production in higher plants. Dordrecht: Springer; 2009. p. 47–63.
[56] Grauda D, Mikelsone A, Lisina N, et al. Anther culture effectiveness in producing doubled haploids of cereals. Proceedings of the Latvian Academy of Sciences, Section B: Natural, Exact, and Applied Sciences 2014;68(3–4):142–7. https://doi.org/10.2478/prolas-2014-0016.
[57] Dahleen LS. Improved plant regeneration from barley callus cultures by increased copper levels. Plant Cell, Tiss Organ Cult 1995;43(3):267–9.
[58] Purnhauser L, Gyulai G. Effect of copper on shoot and root regeneration in wheat, triticale. rape and tobacco tissue cultures Plant Cell Tiss Organ Cult 1993;35(2):131–9. https://doi.org/10.1007/bf00032962.
[59] Datta SK. Androgenic haploids: Factors controlling development and its application in crop improvement. Curr Sci 2005;89(11):1870–8.
[60] Tyankova N, Zagorska N. Factors affecting in vitro androgenesis in cereals. Int J Plant Dev Biol 2008;2(1):59–78.
[61] Nuutila AM, Hämäläinen J, Mannonen L. Optimization of media nitrogen and copper concentrations for regeneration of green plants from polyembryogenic cultures of barley (*Hordeum vulgare* L.). Plant Sci 2000;151:85–92. https://doi.org/10.1016/S0168-9452(99)00202-2.
[62] Brew-Appiah RAT, Ankrah N, Liu W, et al. Generation of doubled haploid transgenic wheat lines by microspore transformation. PLoS ONE 2013;8(11):e80155. https://doi.org/10.1371/journal.pone.0080155. 24260351.
[63] Mirzaei M, Kahrizi D, Rezaeizad A. Androgenesis and spontaneous chromosome doubling in *Hordeum vulgare* L. Researches of the First International Conference. Babylon and Razi Universities; 2011. p. 248–52.
[64] Kumlehn J, Serazetdinova L, Hensel G, et al. Genetic transformation of barley (*Hordeum vulgare* L.) via infection of androgenetic pollen cultures with *Agrobacterium tumefaciens*. Plant Biotechnol J 2006;4(2):251–61. https://doi.org/10.1111/j.1467-7652.2005.00178.x. 17177801.
[65] Wang P, Chen Y. Preliminary study on production of high of pollen H2 generation in winter wheat grown in the field. Acta Agron Sin 1983;9:283–4.
[66] Zhuang JJ, Xu J. Increasing differentiation frequencies in wheat pollen callus. In: Hu H, Vega MR, editors. Cell and tissue culture techniques for cereal crop improvement. Beijing: Science Press; 1983.