The action of the Cu2+, Ag+ and time inducing the in vitro anther culture-derived barley regenerants

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Abstract

Background

Plant regeneration via anther cultures is a world-wide approach as it allows for the regeneration of uniform and homozygous double haploids. Recent studies have shown that in vitro cultures are the origin of the so-called tissue culture-induced variation (TCIV) that may lead to off-type regenerants. Moreover, the regeneration of green plants may be limited by the presence of albinos. It was demonstrated that the presence of Cu$^{2+}$ and Ag$^+$ ions in the regeneration medium might increase the number of green plants.

Results

DArTseqMet markers were evaluated based on regenerants and donor plants derived via in vitro anther cultures of barley. The regenerants were obtained under varying Cu$^{2+}$ and Ag$^+$ ion concentration in the regeneration medium during distinct time conditions of the tissue cultures. The DArTseqMet markers were quantified using a semi-quantitative MSAP approach delivering data on CG and CHG sequence contexts de novo methylation and demethylation. Under each tissue culture conditions, the number of regenerated green plants per 100 anthers was evaluated. Conditional moderation analysis was applied to test for the role of Cu$^{2+}$ and Ag$^+$ ions in the medium. Moreover, the importance of the time of in vitro anther cultures were analyzed.

Conclusions

Our data demonstrate that DNA de novo methylation and demethylation affecting CG and CXG DNA sequence contexts is moderated by the presence of Cu$^{2+}$ and Ag$^+$ ions in the medium conditional on the time of in vitro tissue cultures. The level of de novo methylation and demethylation and the difference between the two is essential for the understanding of moderation. Moreover, Cu$^{2+}$ and Ag$^+$ play in concert moderating DNA methylation changes. For the in vitro tissue culture purposes, the lower the delta value equal to de novo methylation less demethylation and the higher the value of the (Cu+Ag) predictor conditional on time, the higher the number of green plants should be evaluated. Moreover, evaluation of GPs is even more probable under positive delta and higher (Cu+Ag) values.
Our data are congruent with the putative function of these ions in the ethylene and DNA methylation pathways.

Background

An *in vitro* plant regeneration via anther cultures requires cell reprogramming [1], including DNA demethylation and *de novo* methylation of cytosine residues [2] accruing in symmetric (CG and CHG) and asymmetric (CHH) methylation contexts [3, 4]. The methylation of symmetric CG sequence contexts is performed during DNA replication cycle [5, 6], whereas CHG sequence methylation changes are controlled by genetic and epigenetic mechanisms [7-10]. The asymmetric methylation change affecting CHH sites is regulated by epigenetic mechanisms [11] related to stressful conditions influencing plants [12].

Plant regeneration via anther cultures requires precise tuning of the *in vitro* tissue culture conditions, including the concentration of ingredients (i.e., the balanced concentration of ions) that may influence cellular processes promoting plant regeneration. Such ingredients may encourage induction of cell reprogramming [13-16] and potentially change the balance of biochemical pathways. For example, the addition of Cu\(^{2+}\) ions may affect mitochondrial Complex IV [17] belonging to the electron transport chain and is involved in the copper-delivery pathway and creates functional ethylene receptors [18]. It may also form complexes with ethylene. Its improper functioning may result in ATP deficiency [19, 20], or burst of reactive oxygen species (ROS) [21]. On the other hand, silver ion regulates the polyamine pool in a plant [22], ethylene- and calcium-mediated pathways [23], and plays a role in the physiological process including morphogenesis and prone the uptake of Ca\(^{2+}\) into a cell [24]. The presence of Ca\(^{2+}\) may be a stress signal for the nucleus [25]. If the uptake of Ca\(^{2+}\) ions due to the presence of Ag\(^{+}\) in the medium precedes stressful conditions, then burst of Ca\(^{2+}\) and ROS from mitochondria [26, 27] and other organelles may be mitigated winning the cell time for reprogramming [28] changing a haploid cell fate for green plant regeneration [29]. The process affects nuclear DNA [1] at the methylation level [2].

To study the DNA methylation changes and their relation with the number of green plants, several
molecular marker systems could be exploited, including metAFLP [30], MSAP [31], and DArT [32]. The first two are based on the AFLP approach [33] but use distinct isoschizomers recognizing different restriction sites and different methylation patterns [34, 35]. A newly developed DArT system (DArTseqMet) that takes advantage of methylation changes due to *Hpa*II and *Msp*I isoschizomers in combination with NGS [36, 37] might also be a method of choice. The results of the DArTseqMet could be used for quantification of the DNA methylation changes if a semi-quantitative MSAP technique is involved [38].

Finally, to study relationships of different factors affecting the given phenomenon, moderation, and mediation analysis could be employed [39]. The approach is mostly used in psychology [40], medicine [41], economy, and business sciences [42]. It allows the identification of moderators or mediators, however, it was hardly used in studies on in vitro tissue cultures. It seems however, that it may have a wide range of applications allowing a better understanding of relations among many factors of biological systems.

Our previous studies have demonstrated that manipulating Cu$^{2+}$, Ag$^{+}$ ion concentration one may optimize *in vitro* anther cultures of barley towards the increased number of green plants [43]. We have also shown that under varying conditions of Cu$^{2+}$, Ag$^{+}$, and time (T) differences in DNA methylation of the symmetric and asymmetric context may appear [44]. According to that results, the role of the time seems to be negligible, whereas the others [45] indicated that the longevity of *in vitro* tissue cultures might influence DNA methylation pattern [46] or even sequence changes [47]. We suspect that the time of *in vitro* tissue cultures may moderate the action of the ions being present in the *in vitro* tissue culture medium resulting in a change of the DNA methylation patterns and affecting the number of green plants regenerated via *in vitro* anther cultures. We also hypothesize that green plant regeneration is the result of low *de novo* methylation and a high level of demethylation. Thus, delta equal to *de novo* methylation less demethylation might be a predictor of such moderation.

Moreover, we cannot exclude that Cu$^{2+}$ and Ag$^{+}$ ions in the regeneration medium act simultaneously stimulating green plant regeneration in anther cultures. The study aims to analyze the role of Cu$^{2+}$,
Ag⁺ ions, and the time in the regeneration of green plants via in vitro anther cultures due to changes in DNA methylation patterns affecting CG and CXG symmetric context.

Results

In vitro anther tissue cultures performed under nine distinct conditions (trials M1-M9) varying in the Cu²⁺, Ag⁺ ion concentrations, and time allowed the regeneration of 35 plants. As indicated earlier [43], no morphological differences among regenerants were observed, and all of them were in the type of an anther tissue donor plant. DNA isolation from fresh leaves of donor and regenerated DH plants using commercial kits resulted in integral samples without impurities. The new generation sequencing approach exploiting HpaII and MspI endonucleases that differ in sensitivity towards site DNA methylation [48] was used to evaluate DArTseqMet DNA markers. The markers were classified to those related to de novo methylation and demethylation within CG and CXG contexts, following the procedure described earlier [38]. The DNA methylation characteristics were evaluated, as indicated in Table S1 (Additional file 1).

A minimum population size required to achieve actual power of statistics more that 0.31 was estimated for 35 ($F(2,31)=2.9113, f^2=0.111$).

Conditional moderation analyses performed for eight analyses (Additional file 1: Table S2, Analysis A-H) showed that all of them were significant. The DAIC values were lower than 2 [49], the relative likelihood of the models varied from 0.88 to 1, whereas Akaike’s weights from 0.1167 to 0.1319, respectively, indicating their nearly equal probability. Six of them were significant in the case of all predictors (Additional file 1: Table S2, Analyses A, B, C, D, E, and G), whereas the others (Additional file 1: Table S2, Analyses F and H) were insignificant for some predictors or interactions. All the analyses were significant when the highest order unconditional interaction ($X^*W^*Z$, where X states for CG_DNM, CXG_DNM, CG_DM, CXG_DM, W for Cu and Ag and Z for Time) was tested. The tested analyses explained 74.3 – 98.5 % of the variance as indicated by $R^2$ of the models A-H. Conditional $X^*W$ interactions at values of Z evaluated for all analyses (Additional file 1: Table S3) indicated the importance of the time of in vitro tissue cultures as a conditional variable.

The conditional moderation analyses (Additional file 1: Fig. S1-S8) have shown that under high concentration of the Cu²⁺ and Ag⁺ ions in the regeneration medium and a long time of in vitro anther culture, the CG and CXG context should not undergo extensive de novo methylation and the higher number of the green plants regenerated per 100 anthers (GPs) is to be expected. Under the same concentration of Cu²⁺ and Ag⁺ and short time of tissue cultures, plant regeneration is also expected. The regenerants should exhibit a low-level of the CG and CXG demethylation. The GPs should also be regenerated under a long time of tissue culture. Such plants would most probably be highly demethylated at the CG and CXG sequence contexts. Thus, under a long and moderate time of the in vitro anther tissue cultures and high concentration of the Cu²⁺ and Ag⁺ ions, the highest number of the GPs should be regenerated. The CG and CXG sequences will exhibit low level de novo methylation
and a high level of demethylation, indicating that such conditions are preferential for the regeneration of the GPs (Table 1). Our results demonstrated that de novo methylation and DNA demethylation affecting CG and CXG sequence contexts were moderated by Cu\(^{2+}\) and Ag\(^{+}\) ions present in the medium conditional on the time of in vitro anther cultures of barley and that such conditions of tissue cultures influenced the number of green plants regenerated per 100 anthers (Table 1).

Table 1 The arrangement of results illustrating the number of the putative green plants derived via anther cultures assuming on the edges of tested conditions (Additional file 1: Fig. S1-S8)
| Sequence context | De novo methylation or demethylation level | Time (days) | Cu²⁺ (µM) | Ag⁺ (µM) |
|------------------|------------------------------------------|-------------|-----------|----------|
|                  |                                          |             | 0.1       | 5        | 10       | 0         | 10        | 60        |
| CG_DNM Low       |                                          | 21          | 1         | 0        | -1       | 0         | -1        | -28       |
|                  |                                          | 28          | -2.5      | 0        | 2.5      | 0         | 0         | 0         |
|                  |                                          | 35          | -6        | 0        | 6        | 0         | 5         | 28        |
|                  | High                                     | 21          | 1.5       | 2        | 2.5      | 0         | 2         | 10        |
|                  |                                          | 28          | 2         | 1.5      | 1        | 0         | 0         | 2         |
|                  |                                          | 35          | 2         | 0        | -2       | 0         | 0         | -1        |
|                  | Low                                      | 21          | 0         | -2.5     | -5       | 0         | 2         | 19        |
|                  |                                          | 28          | -2.5      | -1       | 1        | 0         | 5         | 40        |
|                  |                                          | 35          | -6        | 1        | 8        | 0         | 10        | 60        |
|                  | High                                     | 21          | 1         | 2        | 3        | 0         | 0         | 0         |
|                  |                                          | 28          | 1.5       | 1        | 0.5      | 0         | -1        | -10       |
|                  |                                          | 35          | 4         | 1        | -2       | 0         | -1        | -20       |
| CG_DN M Low      |                                          | 21          | -1        | 1        | 3        | 0         | 3         | 16        |
|                  |                                          | 28          | 2         | 1        | 0.5      | 0         | 1         | 8         |
|                  |                                          | 35          | 3         | 0        | -3       | -1        | 0         | 1         |
|                  | High                                     | 21          | 1         | 0.5      | -0.5     | 0         | -1        | -13       |
|                  |                                          | 28          | -2        | 0.5      | 2        | 1         | 0         | -2        |
|                  |                                          | 35          | -4        | 0        | 4        | 1         | 2         | 9         |
| CXG_DNM Low      |                                          | 21          | 0         | 2.5      | 5        | 0         | 3         | 19        |
|                  |                                          | 28          | 3         | 2        | 0        | 1         | 3         | 7         |
|                  |                                          | 35          | 6         | 1        | -6       | 1         | 0         | -5        |
|                  | High                                     | 21          | 1         | 0        | -1       | 0         | -1        | -10       |
|                  |                                          | 28          | -3        | 0        | 3        | 1         | 1         | -1        |
|                  |                                          | 35          | -5        | 1        | 7.5      | 2         | 3         | 10        |

Conditional moderation analysis indicating the number of GPs derived under certain tissue culture medium conditions (Cu²⁺ and Ag⁺ concentrations) and the time of the in vitro anther cultures. DNM - *de novo* methylation, DM - demethylation; CG and CXG are the DNA sequences that could be *de novo* methylated or demethylated.

Moderation of GPs due to the delta (a predictor) moderated by Cu²⁺ conditional on time (Fig. 1,
Additional file 1: Table S2, Analysis I) shows that short time of the *in vitro* tissue cultures containing Cu$^{2+}$ at the highest concentrations should result in the increased number of green regenerants with the lowest negative delta values. Increasing the time of *in vitro* anther tissue cultures using low Cu$^{2+}$ concentration should lead towards regeneration of GPs (Fig. 1, Table 1). Increasing the time of the anther cultures and keeping the highest concentration of Cu$^{2+}$ seems to be the way to evaluate the regenerants; however, such plants would have the highest positive delta value. The conditional delta*Cu interaction is not valid through 25 day of *in vitro* anther cultures (Additional file 1: Table S12).

Moderation of GPs due to the delta variable moderated by Ag$^+$ conditional on time (Fig. 2, Additional file 1: Table S2, Analysis J) is nearly identical as the one with Cu$^{2+}$. Both models are nearly equally probable as indicated by Akaike’s weigh values (Additional file 1: Table S2, Analysis I and J). Short time of the *in vitro* tissue cultures containing Ag$^+$ at the highest concentrations should result in the increased number of green regenerants with the lowest delta values. Increasing the time of the anther cultures and keeping the highest concentration of Ag$^+$ results in the highest number of regenerants; however, such plants would have the increased positive delta value. The conditional delta*Ag interaction is not significant through 28 day of the *in vitro* anther cultures (Additional file 1: Table S13).

Using combined delta = DNM - DM variable moderated by unified moderator Cu + Ag conditional on the time of the *in vitro* anther tissue cultures, the analysis was significant (Fig. 3, Additional file 1: Table S2, Analysis K). Under a short time of the *in vitro* anther cultures and using Cu + Ag higher than 15, one should expect more green regenerants with a negative value of delta. It means that the number of *de novo* methylation is much lower than the number of demethylation events affecting CG and CXG DNA sequence contexts. When the Cu x Ag variable is large, but the delta is positive (*de novo* methylation exceeds demethylation), then regeneration of GPs is expected under a long time of the *in vitro* tissue cultures. The moderation is not valid through the 28 day of the *in vitro* tissue culture (Additional file 1, Table S14).

**Discussion**

*In vitro* anther tissue cultures is an essential approach for the evaluation of uniform plant materials useful for breeding programs [50-53]. The approach is being world-wide used, but a growing number of data clearly shows that plant regeneration via anther culture is affected by an *in vitro* TCIV [54-57] that could be transmitted to a progeny [58]. Among others, the TCIV is due to changes in the DNA methylation patterns affecting distinct methylation contexts (symmetric and asymmetric) that are under either genetic or epigenetic control [44]. As plant regeneration requires cell reprogramming [1] involving DNA methylation pattern changes, it is of value to understand whether ingredients present
in the *in vitro* medium and other factors such as the time of *in vitro* cultures may affect methylation changes and the number of the regenerated green plants that may be limited by the presence of albinos [59]. Among many components used in the *in vitro* anther culture, Cu\(^{2+}\) and Ag\(^{+}\) are being considered as promising [60, 61]. There are shreds of evidence that they may increase the number of green regenerants in cereals [62, 63]. However, the putative role of the ions is not explicit.

Moreover, the action of Cu\(^{2+}\) and Ag\(^{+}\) ions might be modulated by the time of tissue cultures, influencing DNA methylation level [46]. DNA methylation pattern changes in tissue cultures may regulate the level of green plant regeneration in the result of the cell reprogramming process [64]. Our data demonstrate that Cu\(^{2+}\) and Ag\(^{+}\) ions conditional on the time of *in vitro* anther cultures of barley moderate the level of CG and CXG DNA sequence contexts of *de novo* methylation and demethylation resulting in a distinct number of green plants. In general, the higher the level of demethylation and the lower the level of *de novo* methylation of the symmetric contexts, the higher the number of green regenerants. Based on conditional moderation analysis of the quantitative characteristics of DNA methylation contexts evaluated on DArTseqMet markers quantified by the MSAP approach [38] we have shown that long time of *in vitro* anther tissue cultures with a high concentration of Cu\(^{2+}\) and Ag\(^{+}\) ions increases the GPs number with a low level of CG sequence context *de novo* methylation (Additional file 1: Fig. S1-S2; Table S2, Analyses A, B; Table S4, S5).

Moderation of the GPs due to the *de novo* methylation of the CXG DNA sequence context by Cu\(^{2+}\) conditional on the time (Additional file1: Fig. S3; Table S2, Analysis C, Table S6) shows that the longer the time of *in vitro* anther culture and the higher the concentration of the Cu\(^{2+}\) ions the greater the number of the GPs. A long time of the *in vitro* anther tissue culture with a high concentration of Ag\(^{+}\) ions in regeneration medium should promote regeneration of the GPs with a low level of *de novo* methylation events affecting CXG sites (Additional file 1: Fig. S4, Table S2, Analysis D; Table S7).

A short time of the *in vitro* anther tissue cultures with a high concentration of Cu\(^{2+}\) and Ag\(^{+}\) ions in the regeneration medium results in the increased number of GPs with a low level of demethylation of
the CXG sequences, whereas the long time of the in vitro tissue cultures should lead towards the increasing number of GPs with a high level of DNA demethylation of the CXG sites (Additional file1: Fig. S5; Table S2, Analysis E; Table S8). A long time of the in vitro anther tissue cultures with a high concentration of Cu$^{2+}$ and Ag$^+$ ions in the regeneration medium should lead towards an increased number of GPs with the highest level of DNA demethylation affecting CG sites. Contrary, a short time of in vitro tissue cultures and low concentration of Cu$^{2+}$ and Ag$^+$ ions should decrease in the number of GPs. The regenerants should have a low level of CXG sequences demethylation (Additional file1: Fig. S6-S8; Table S2, Analyses F-H; Table S9-S11).

Our data are in agreement with prior studies suggesting that for the regeneration of green plants, cell reprogramming [65] is needed. We have demonstrated that delta equal to de novo methylation less demethylation (all symmetric contexts taken together) may be used as a predictor in moderation conditional on time. As delta was calculated without focusing on any of the methylation contexts (Fig. 1 and 2, Additional file 1: Table S2, Analyses I and J; Table S12-S13), and any conditional moderation analyses (Additional file 1: Table S2, Analyses A-H) concerning CG and CXG sequences moderated by Cu$^{2+}$ and/or Ag$^+$ ions were equally probable (as indicated by Akaike’s weight values) (Additional file 1: Table S2), we tend to think that the demethylation and de novo DNA methylation affect all types of symmetric sites. No preferences towards any of the sequence types to methylation change, involving a broad spectrum of active mechanisms responsible for DNA demethylation [66] and de novo DNA methylation [67] that play in concert was observed.

Interestingly, plant regeneration takes place when delta = DNM-DM is negative; demethylation events are either higher than or precede de novo methylation, and such a sequence of changes is vital for plant regeneration in anther cultures. The regeneration of GPs is also promoted when delta is positive under the highest (Ag + Cu) concentration conditional on a long time of in vitro tissue cultures (Fig. 3, Additional file 1: Table S2, Analysis K; Table S14). Moderation analysis shows that there is a window of time when GPs regeneration is hardly possible, but even under positive delta (when de novo methylation exceeds DNA demethylation), regeneration may still be significant in barley anther
cultures. The presented data may be interpreted in terms of full cell reprogramming to regenerate under a long time of \textit{in vitro} tissue culture conditions that require increased (Ag + Cu) concentration. The molecular basis of Ag$^+$ and Cu$^{2+}$ ions in tissue cultures are lacking; however, they evidently “cooperate” with each other affecting the number of GPs conditional on the time of \textit{in vitro} tissue cultures. Our analysis has not only shown that the delta equal to \textit{de novo} methylation less demethylation could be used as a predictor in conditional moderation analysis, but the sum of Cu$^{2+}$ and Ag$^+$ is also indicative here. We have noticed that the two ions play in concert and moderate DNA methylation in a similar manner resulting in GPs conditional on the time of tissue cultures (Fig. 3, Additional file 1: Table S2, Analysis K, Table S14). The cooperative effect of silver and copper ions could be explained by silver ions substitution for copper ions [68], interfering with ethylene pathways contributing to the regulatory pathways of DNA methylation [69].

Conclusions
Our results reveal that the ions present in the medium of \textit{in vitro} anther cultures of barley moderate distinct DNA methylation context conditional on the time of \textit{in vitro} tissue cultures leading to green regenerants with varying levels of methylation. Thus, optimizing \textit{in vitro} tissue cultures, particular caution is needed to control the level of the \textit{in vitro} tissue culture-induced methylation changes as they may affect the number of green plants. It should be stressed, however, that the actual power of moderation analysis was not high. Further studies are needed to identified cellular compounds that may moderate DNA methylation changes affecting GPs number to understand plant regeneration via anther cultures better.

Methods
Plant materials (spring barley cultivar NAD2 was provided by Poznan Plant Breeders LTD-Nagradowice, Poland) were obtained, as described earlier [43]. Briefly, spikes of donor doubled haploid (DH) plants (D) of barley were used to regenerate new DH plants under varying conditions of Cu$^{2+}$ and Ag$^+$ ion concentrations and time of tissue cultures. Nine of such trials were performed (M1-M9), and the number of green plants regenerated per 100 anthers (GPs) within each trial was evaluated.
DNA isolation was performed from fresh leaves of donor and regenerated plants using the DNeasy MiniPrep kit (Qiagen). DArTseqMet was conducted in DArT PL company (DArTseqMet, developed by Diversity Arrays Technology, https://www.diversityarrays.com/technology-and-resources/dartseq). DArTSeqMet markers were converted into quantitative methylation characteristics following the MSAP approach described earlier [38].

**Power of the statistics**

The minimum population size was calculated in G-Power software [70]. Squared multiple correlation $\rho^2$ was set to 0.1 to calculate effect size $\hat{\rho}^2$ at $a=0.05$ with three predictors and power $(1 - b \; \text{err prob})$ set to 0.31.

**Moderation analysis**

Moderation analysis was conducted in SPSS software V. 26 (https://www.ibm.com/support/pages/node/874712) using A. F. Hayes Process v. 3.4 macro [71]. Conditional moderation model (Fig. 4) [71] was tested. Model quality was tested using second-order Akaike’s Information Criterion for small sample size ($n/k < 40$), where $n$ is a sample size, $k$ states for model parameters and log-likelihood is a measure of model fit using the formula: $AIC = -2(\log\text{-likelihood}) + 2k + 2k^*(k+1)/(n-k-1)$. AIC scores were reported as $\Delta AIC$ (the relative difference between the best model which has a DAIC of zero) using the following formula: $\Delta AIC = AIC_i - \text{min } AIC$, where $AIC_i$ is the AIC of the $i$ model and min AIC is the score for the best model. If DAIC was less than 2, the model was assumed substantial. Akaike weights were used in model averaging. They represent the relative likelihood of a model. For each model, the relative likelihood (RL) of the model, which is $\exp(-0.5 * \Delta AIC$ score for that model), was calculated. The Akaike weight (AW) for a model is the $\exp(-0.5 * \Delta AIC$ score for that model) value divided by the sum of all such values across all models [49] for the given type of models.

**Supplementary Information**

**Additional file 1:**

**Table S1** The arrangement of the MSAP DNA methylation characteristics for regenerants obtained via *in vitro* anther culture
**Table S2** The arrangement of statistics for conditional moderation analyses based on model 3. RL - relative likelihood, AW - Akaike's weight, LLCI – ULCI is a 95% confidence interval

**Table S3** The arrangement of conditional X*W interaction at values of Z

**Table S4** Conditional CG_DNM*Cu interaction at values of the moderator Time. For the analysis

PROCESS macro v. 3.4 by A.F. Hayes was used

**Table S5** Conditional CG_DNM*Ag interaction at values of the moderator Time. For the analysis

PROCESS macro v. 3.4 by A.F. Hayes was used

**Table S6** Conditional CXG_DNM*Cu interaction at values of the moderator Time. For the analysis

PROCESS macro v. 3.4 by A.F. Hayes was used

**Table S7** Conditional CXG_DNM*Ag interaction at values of the moderator Time. For the analysis

PROCESS macro v. 3.4 by A.F. Hayes was used

**Table S8** Conditional CG_DM*Cu interaction at values of the moderator Time. For the analysis

PROCESS macro v. 3.4 by A.F. Hayes was used

**Table S9** Conditional CG_DM*Ag interaction at values of the moderator Time. For the analysis

PROCESS macro v. 3.4 by A.F. Hayes was used

**Table S10** Conditional CXG_DM*Cu interaction at values of the moderator Time. For the analysis

PROCESS macro v. 3.4 by A.F. Hayes was used

**Table S11** Conditional CXG_DM*Ag interaction at values of the moderator Time. For the analysis

PROCESS macro v. 3.4 by A.F. Hayes was used

**Table S12** Conditional delta*Cu interaction at values of the moderator Time. For the analysis

PROCESS macro v. 3.4 by A.F. Hayes was used

**Table S13** Conditional delta*Ag interaction at values of the moderator Time. For the analysis

PROCESS macro v. 3.4 by A.F. Hayes was used

**Table S14** Conditional delta*(Ag+Cu) interaction at values of the moderator Time. For the analysis

PROCESS macro v. 3.4 by A.F. Hayes was used

**Fig. S1** Conditional effect of the focal predictor (Model 3). GP100Ant – green plants per 100 anthers, CG_DNM – *de novo* methylation of the CG contexts. Variables: Cu^{2+} – W, Time – Z
**Fig. S2** Conditional effect of the focal predictor (Model 3). GP100Ant – green plants per 100 anthers, CG_DNM – *de novo* methylation of the CG contexts. Variables: Ag⁺ – W, Time – Z

**Fig. S3** Conditional effect of the focal predictor (Model 3). GP100Ant – green plants per 100 anthers, CXG_DNM – *de novo* methylation of the CXG contexts. Variables: Cu²⁺ – W, Time – Z

**Fig. S4** Conditional effect of the focal predictor (Model 3). GP100Ant – green plants per 100 anthers, CXG_DNM – *de novo* methylation of the CXG contexts. Variables: Ag⁺ – W, Time – Z

**Fig. S5** Conditional effect of the focal predictor (Model 3). GP100Ant – green plants per 100 anthers, CG_DMV – demethylation of the CG contexts. Variables: Cu²⁺ – W, Time – Z

**Fig. S6** Conditional effect of the focal predictor (Model 3). GP100Ant – green plants per 100 spikes, CG_DMV – demethylation of the CG contexts. Variables: Ag⁺ – W, Time – Z

**Fig. S7** Conditional effect of the focal predictor (Model 3). GP100Ant – green plants per 100 anthers, CXG_DMV – demethylation of the CXG contexts. Variables: Cu²⁺ – W, Time – Z

**Fig. S8** Conditional effect of the focal predictor. GP100Ant – green plants per 100 anthers, CXG_DMV – demethylation of the CXG contexts. Variables: Ag⁺ – W, Time – Z

**Abbreviations**

TCIV: Tissue culture-induced variation; ROS: Reactive oxygen species; GPs: green plants regenerated per 100 anthers

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding
author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' contributions**

PTB and RO conceived and designed the research. RO conducted the experiments. PTB performed statistical analyzes. PTB and RO analyzed the generated data and wrote the manuscript. The final version of the manuscript was edited and approved by both the authors.

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Figure 1

Conditional effect of the focal predictor. GP100Ant - green plants per 100 anthers is a dependent variable, delta - (CG_DNM+CXG_DNM)-(CG_DM+CXG_DM) is an independent variable whereas Cu2+ is a moderator conditional on the time of in vitro anther cultures
Figure 1

Conditional effect of the focal predictor. GP100Ant - green plants per 100 anthers is a dependent variable, delta - (CG_DNM+CXG_DNM)-(CG_DM+CXG_DM) is an independent variable whereas Cu2+ is a moderator conditional on the time of in vitro anther cultures.
Figure 2

Conditional effect of the focal predictor. GP100Ant - green plants per 100 anthers is a dependent variable, delta - (CG_DNM+CXG_DNM)-(CG_DM+CXG_DM) is an independent variable whereas Ag+ is a moderator conditional on the time of in vitro anther cultures.
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Figure 3

Conditional effect of the focal predictor. GP100Ant - green plants per 100 anthers is a dependent variable, delta - (CG_DNM+CXG_DNM)-(CG_DM+CXG_DM) is an independent variable whereas (Cu +Ag) is a moderator conditional on the time of in vitro anther cultures
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Figure 4

Schematic illustration of conditional moderation model. X is a predictor, Y – dependent variable, W – moderator conditional on Z
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