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 $Cu^{2+,}$ 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 *Hpa*II and *Msp*I 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 Cu^{2+} 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 Cu^{2+} 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 Cu^{2+} 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)	Cu^{2+} (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
CXG_DNM	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_DM	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_DM	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 (Cu^{2+} 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 Cu^{2+} conditional on time (Fig. 1, Additional file1: 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 *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 *in vitro* tissue cultures. Our analysis has not only shown that the delta equal to *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 *in vitro* anther cultures of barley moderate distinct DNA methylation context conditional on the time of *in vitro* tissue cultures leading to green regenerants with varying levels of methylation. Thus, optimizing *in vitro* tissue cultures, particular caution is needed to control the level of the *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 p^2 was set to 0.1 to calculate effect size f^2 at a=0.05 with three predictors and power (1 - *b* 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-likelihood) + 2k + 2k*(k+1)/(n-k-1). AIC scores were reported as ΔAIC (the relative difference between the best model which has a DAIC of zero) using the following formula: ΔAIC = AICi – min AIC, where AICi 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 * ΔAIC score for that model), was calculated. The Akaike weight (AW) for a model is the exp(-0.5 * ΔAIC 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: Cu^{2+} – 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: Cu^{2+} – 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: Cu^{2+} – 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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References

1. Testillano PS: **Microspore embryogenesis: targeting the determinant factors of stress-induced cell reprogramming for crop improvement**. *Journal of Experimental Botany* 2019, **70**(11):2965-2978.
2. El-Tantawy AA, Solís MT, Risueño MC, Testillano PS: **Changes in DNA Methylation Levels and Nuclear Distribution Patterns after Microspore Reprogramming to Embryogenesis in Barley**. *Cytogenetic and Genome Research* 2014, **143**(1-3):200-208.
3. Bartels A, Han Q, Nair P, Stacey L, Gaynier H, Mosley M, Huang QQ, Pearson JK, Hsieh T-F, An Y-QC *et al*: **Dynamic DNA Methylation in Plant Growth and Development**. *International journal of molecular sciences* 2018, **19**(7):2144.
4. Henderson IR, Jacobsen SE: **Epigenetic inheritance in plants**. *Nature* 2007, **447**(7143):418-424.
5. Jullien PE, Kinoshita T, Ohad N, Berger F: **Maintenance of DNA Methylation during the Arabidopsis Life Cycle Is Essential for Parental Imprinting**. *The Plant Cell* 2006, **18**(6):1360-1372.
6. Law JA, Jacobsen SE: **Establishing, maintaining and modifying DNA methylation patterns in plants and animals**. *Nature Reviews Genetics* 2010, **11**:204.
7. Lindroth AM, Cao X, Jackson JP, Zilberman D, McCallum CM, Henikoff S, Jacobsen SE: **Requirement of CHROMOMETHYLASE3 for Maintenance of CpXpG Methylation**. *Science* 2001, **292**(5524):2077-2080.
8. Cao X, Jacobsen SE: **Role of the Arabidopsis DRM Methyltransferases in De Novo DNA Methylation and Gene Silencing**. *Current Biology* 2002, **12**(13):1138-1144.

9. Lindroth AM, Shultis D, Jasencakova Z, Fuchs J, Johnson L, Schubert D, Patnaik D, Pradhan S, Goodrich J, Schubert I *et al*: **Dual histone H3 methylation marks at lysines 9 and 27 required for interaction with CHROMOMETHYLASE3**. *The EMBO journal* 2004, **23**(21):4286-4296.
10. Mathieu O, Reinders J, Caikovski M, Smathajitt C, Paszkowski J: **Transgenerational Stability of the *Arabidopsis* Epigenome Is Coordinated by CG Methylation**. *Cell* 2007, **130**: 851–862.
11. Cao X, Jacobsen SE: **Locus-specific control of asymmetric and CpNpG methylation by the DRM and CMT3 methyltransferase genes**. *Proc Natl Acad Sci U S A* 2002, **99 Suppl 4**:16491-16498.
12. Bednarek PT, Orłowska R: **Plant tissue culture environment as a switch-key of (epi)genetic changes**. *Plant Cell, Tissue and Organ Culture (PCTOC)* 2020, **140**(2):245-257.
13. Pěnčík A, Turečková V, Paulišić S, Rolčík J, Strnad M, Mihaljević S: **Ammonium regulates embryogenic potential in Cucurbita pepo through pH-mediated changes in endogenous auxin and abscisic acid**. *Plant Cell, Tissue and Organ Culture (PCTOC)* 2015, **122**(1):89-100.
14. Bustillo-Avendaño E, Ibáñez S, Sanz O, Sousa Barros JA, Gude I, Perianez-Rodriguez J, Micol JL, Del Pozo JC, Moreno-Risueno MA, Pérez-Pérez JM: **Regulation of Hormonal Control, Cell Reprogramming, and Patterning during De Novo Root Organogenesis**. *Plant Physiology* 2018, **176**(2):1709-1727.
15. Kiyosue T, Takano K, Kamada H, Harada H: **Induction of somatic embryogenesis in carrot by heavy metal ions**. *Canadian Journal of Botany* 1990, **68**(10):2301-2303.
16. Ikeda-Iwai M, Umehara M, Satoh S, Kamada H: **Stress-induced somatic embryogenesis in vegetative tissues of Arabidopsis thaliana**. *The Plant Journal* 2003, **34**(1):107-114.
17. Mansilla N, Racca S, Gras DE, Gonzalez DH, Welchen E: **The Complexity of Mitochondrial Complex IV: An Update of Cytochrome c Oxidase Biogenesis in Plants**. *International journal of molecular sciences* 2018, **19**(3):662.
18. Wang KLC, Li H, Ecker JR: **Ethylene biosynthesis and signaling networks**. *The Plant cell* 2002, **14 Suppl**(Suppl):S131-S151.
19. Zhu Q, Dugardeyn J, Zhang C, Takenaka M, Kühn K, Craddock C, Smalle J, Karampelias M, Denecke J, Peters J *et al*: **SLO2, a mitochondrial pentatricopeptide repeat protein affecting several RNA editing sites, is required for energy metabolism**. *The Plant Journal* 2012, **71**(5):836-849.
20. Geisler DA, Päpke C, Obata T, Nunes-Nesi A, Matthes A, Schneitz K, Maximova E, Araújo WL, Fernie AR, Persson S: **Downregulation of the δ-subunit reduces mitochondrial ATP synthase levels, alters respiration, and restricts growth and gametophyte development in Arabidopsis**. *The Plant cell* 2012, **24**(7):2792-2811.
21. Møller IM: **PLANT MITOCHONDRIA AND OXIDATIVE STRESS: Electron Transport, NADPH Turnover, and Metabolism of Reactive Oxygen Species**. *Annual Review of Plant Physiology and Plant Molecular Biology* 2001, **52**(1):561-591.
22. Bush DS: **Calcium Regulation in Plant Cells and its Role in Signaling**. *Annual Review of Plant Physiology and Plant Molecular Biology* 1995, **46**(1):95-122.

23. Kumar V, Parvatam G, Ravishankar GA: **AgNO3 - a potential regulator of ethylene activity and plant growth modulator**. *Electronic Journal of Biotechnology* 2009, **12**(2):0-0.
24. Klíma P, Laňková M, Vandenbussche F, Van Der Straeten D, Petrášek J: **Silver ions increase plasma membrane permeability through modulation of intracellular calcium levels in tobacco BY-2 cells**. *Plant Cell Reports* 2018, **37**(5):809-818.
25. Tuteja N, Mahajan S: **Calcium signaling network in plants: an overview**. *Plant signaling & behavior* 2007, **2**(2):79-85.
26. Feissner RF, Skalska J, Gaum WE, Sheu S-S: **Crosstalk signaling between mitochondrial Ca2+ and ROS**. *Front Biosci (Landmark Ed)* 2009, **14**:1197-1218.
27. Görlach A, Bertram K, Hudecova S, Krizanova O: **Calcium and ROS: A mutual interplay**. *Redox Biology* 2015, **6**:260-271.
28. Testillano PS, Coronado MJ, Seguí JM, Domenech J, González-Melendi P, Raška I, Risueño MC: **Defined Nuclear Changes Accompany the Reprogramming of the Microspore to Embryogenesis**. *Journal of Structural Biology* 2000, **129**(2):223-232.
29. Islam SM, Tuteja N: **Enhancement of androgenesis by abiotic stress and other pretreatments in major crop species**. *Plant Sci* 2012, **182**:134-144.
30. 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):279-292.
31. Baranek M, Cechova J, Kovacs T, Eichmeier A, Wang S, Raddova J, Necas T, Ye X: **Use of Combined MSAP and NGS Techniques to Identify Differentially Methylated Regions in Somaclones: A Case Study of Two Stable Somatic Wheat Mutants**. *PLoS One* 2016, **11**(10):e0165749.
32. Vandenbroucke H, Mournet P, Vignes H, Chaïr H, Malapa R, Duval MF, Lebot V: **Somaclonal variants of taro (*Colocasia esculenta* Schott) and yam (*Dioscorea alata* L.) are incorporated into farmers' varietal portfolios in Vanuatu**. *Genet Resour Crop Evol* 2016, **63**(3):495-511.
33. Vos P, Hogers R, Bleeker M, Reijans M, Lee T, van de. H, M.,, Frijters A, Pot J, Peleman J, Kuiper M *et al*: **AFLP: a new technique for DNA fingerprinting**. *Nucleic Acids Research* 1995, **23**:4407-4414.
34. Bednarek PT, Orłowska R, Koebner RMD, Zimny J: **Quantification of the tissue-culture induced variation in barley (*Hordeum vulgare* L.)**. *BMC Plant Biology* 2007, **7:10**.
35. Machczyńska J, Orłowska R, Zimny J, Bednarek PT: **Extended metAFLP approach in studies of the tissue culture induced variation (TCIV) in case of triticale**. *Molecular Breeding* 2014, **34**:845-854.
36. Jaccoud D, Peng K, Feinstein D, Kilian A: **Diversity arrays: a solid state technology for sequence information independent genotyping**. *Nucleic acids research* 2001, **29**(4):E25-E25.
37. Sansaloni C, Petroli C, Jaccoud D, Carling J, Detering F, Grattapaglia D, Kilian A: **Diversity Arrays Technology (DArT) and next-generation sequencing combined: genome-wide, high throughput, highly informative genotyping for molecular breeding of Eucalyptus**. *BMC Proc* 2011, **5**(Suppl 7):P54-P54.

38. Bednarek PT, Orłowska R, Niedziela A: **A relative quantitative Methylation-Sensitive Amplified Polymorphism (MSAP) method for the analysis of abiotic stress**. *BMC Plant Biol* 2017, **17**(1):79.
39. Hayes AF: **Introduction to Mediation, Moderation, and Conditional Process Analysis. A Regression Bases Approach.** New York, NY, US: A Division of Guilford Publications, Inc.; 2018.
40. MacKinnon DP, Luecken LJ: **How and for whom? Mediation and moderation in health psychology**. *Health Psychol* 2008, **27**(2S):S99-S100.
41. Holroyd KA, Labus JS, Carlson B: **Moderation and mediation in the psychological and drug treatment of chronic tension-type headache: the role of disorder severity and psychiatric comorbidity**. *Pain* 2009, **143**(3):213-222.
42. Namazi M, Namazi N-R: **Conceptual Analysis of Moderator and Mediator Variables in Business Research**. *Procedia Economics and Finance* 2016, **36**:540-554.
43. Orłowska R, Pachota KA, Machczyńska J, Niedziela A, Makowska K, Zimny J, Bednarek PT: **Improvement of anther cultures conditions using the Taguchi method in three cereal crops**. *Electronic Journal of Biotechnology* 2020, **43**:8-15.
44. Orłowska R, Bednarek PT: **Precise evaluation of tissue culture-induced variation during optimisation of in vitro regeneration regime in barley**. *Plant Mol Biol* 2020.
45. Rodríguez López CM, Wetten AC, Wilkinson MJ: **Progressive erosion of genetic and epigenetic variation in callus-derived cocoa (Theobroma cacao) plants**. *New Phytologist* 2010, **186**(4):856-868.
46. Rival A, Ilbert P, Labeyrie A, Torres E, Doulbeau S, Personne A, Dussert S, Beule´ T, Durand-Gasselin T, Tregear JW *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**:359-368.
47. Bordallo PN, Silva, D.H., Maria, J., Cruz, C.D., Fontes, E.P.: **Somaclonal variation on *in vitro* callus culture potato cultivars**. *Horticultura Brasileira* 2004, **22**(2):300-304.
48. Xiong LZ, Xu CG, Saghai Maroof MA, Zhang Q: **Patterns of cytosine methylation in an elite rice hybrid and its parental lines, detected by a methylation-sensitive amplifcation polymorphism technique**. *Mol Gen Genet* 1999, **261**:439-446.
49. Burnham KP, Anderson DR: **Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach**. New York: Springer Science & Business Media; 2003.
50. Tenhola-Roininen T, Immonen S, Tanhuanpää P: **Rye doubled haploids as a research and breeding tool - a practical point of view**. *Plant Breed* 2006, **125**(6):584-590.
51. Ślusarkiewicz-Jarzina A, Pudelska H, Wozna J, Pniewski T: **Improved production of doubled haploids of winter and spring triticale hybrids via combination of colchicine treatments on anthers and regenerated plants**. *J Appl Genet* 2017, **58**(3):287-295.
52. El-Hennawy MA, Abdalla AF, Shafey SA, Al-Ashkar IM: **Production of doubled haploid wheat lines (Triticum aestivum L.) using anther culture technique**. *Annals of Agricultural Sciences* 2011, **56**(2):63-72.

53. Ohnoutkova L, Vlcko T, Ayalew M: **Barley anther culture**. In: *Methods in Molecular Biology.* vol. 1900: Humana Press Inc.; 2019: 37-52.
54. Machczyńska J, Orłowska R, Mańkowski DR, Zimny J, Bednarek PT: **DNA methylation changes in triticale due to in vitro culture plant regeneration and consecutive reproduction**. *Plant Cell Tiss Organ Cult* 2014, **119**(2):289-299.
55. Orłowska R, Machczyńska J, Oleszczuk S, Zimny J, Bednarek PT: **DNA methylation changes and TE activity induced in tissue cultures of barley (*Hordeum vulgare* L.)**. *Journal of biological research (Thessalonike, Greece)* 2016, **23**:19-19.
56. Fiuk A, Bednarek PT, Rybczyński JJ: **Flow Cytometry, HPLC-RP, and metAFLP Analyses to Assess Genetic Variability in Somatic Embryo-Derived Plantlets of *Gentiana pannonica* Scop.** *Plant Molecular Biology Reporter* 2010, **28**:413-420.
57. Coronel CJ, González AI, Ruiz ML, Polanco C: **Analysis of somaclonal variation in transgenic and regenerated plants of Arabidopsis thaliana using methylation related metAFLP and TMD markers**. *Plant Cell Reports* 2018, **37**(1):137-152.
58. Han Z, Crisp PA, Stelpflug S, Kaeppler SM, Li Q, Springer NM: **Heritable Epigenomic Changes to the Maize Methylome Resulting from Tissue Culture**. *Genetics* 2018, **209**(4):983-995.
59. Makowska K, Oleszczuk S: **Albinism in barley androgenesis**. *Plant Cell Rep* 2014, **33**(3):385-392.
60. Jacquard C, Nolin F, Hecart C, Grauda D, Rashal I, Dhondt-Cordelier S, Sangwan RS, Devaux P, Mazeyrat-Gourbeyre F, Clement C: **Microspore embryogenesis and programmed cell death in barley: effects of copper on albinism in recalcitrant cultivars**. *Plant Cell Rep* 2009, **28**(9):1329-1339.
61. Fei S, Read PE, Riordan TP: **Improvement of embryogenic callus induction and shoot regeneration of buffalograss by silver nitrate**. *Plant Cell, Tissue and Organ Culture* 2000, **60**(3):197-203.
62. Nuutila AM, Hämäläinen J, Mannonen L: **Optimization of media nitrogen and copper concentrations for regeneration of green plants from polyembryogenic cultures of barley (*Hordeum ulgare* L.)**. *Plant Science* 2000, **151**:85-92.
63. Haque M, Siddique AB, Shahinul Islam SM: **Effect of silver nitrate and amino acids on high frequency plants regeneration in barley (*Hordeum vulgare* L.)**. *Plant Tissue Cult Biotechnol* 2015, **25**:37-50.
64. Solís M-T, El-Tantawy A-A, Cano V, Risueño MC, Testillano PS: **5-azacytidine promotes microspore embryogenesis initiation by decreasing global DNA methylation, but prevents subsequent embryo development in rapeseed and barley**. *Frontiers in Plant Science* 2015, **6**(472).
65. Ikeuchi M, Shibata M, Rymen B, Iwase A, Bågman AM, Watt L, Coleman D, Favero DS, Takahashi T, Ahnert SE *et al*: **A Gene Regulatory Network for Cellular Reprogramming in Plant Regeneration**. *Plant Cell Physiol* 2018, **59**(4):765-777.
66. Li Y, Kumar S, Qian W: **Active DNA demethylation: mechanism and role in plant development**. *Plant cell reports* 2018, **37**(1):77-85.
67. He X-J, Chen T, Zhu J-K: **Regulation and function of DNA methylation in plants and animals**. *Cell Res* 2011, **21**(3):442-465.

68. Beyer EM: **A potent inhibitor of ethylene action in plants**. *Plant Physiol* 1976, **58**(3):268-271.
69. Zuo J, Wang Y, Zhu B, Luo Y, Wang Q, Gao L: **Comparative Analysis of DNA Methylation Reveals Specific Regulations on Ethylene Pathway in Tomato Fruit**. *Genes (Basel)* 2018, **9**(5).
70. Faul F, Erdfelder E, Lang A-G, Buchner A: **G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences**. *Behavior Research Methods* 2007, **39**(2):175-191.
71. Hayes A, F: **Introduction to Mediation, Moderation, and Conditional Process Analysis. A Regression-Based Approach.**, Seconfd edition edn. New York: The Guilford Press; 2017.

Figures

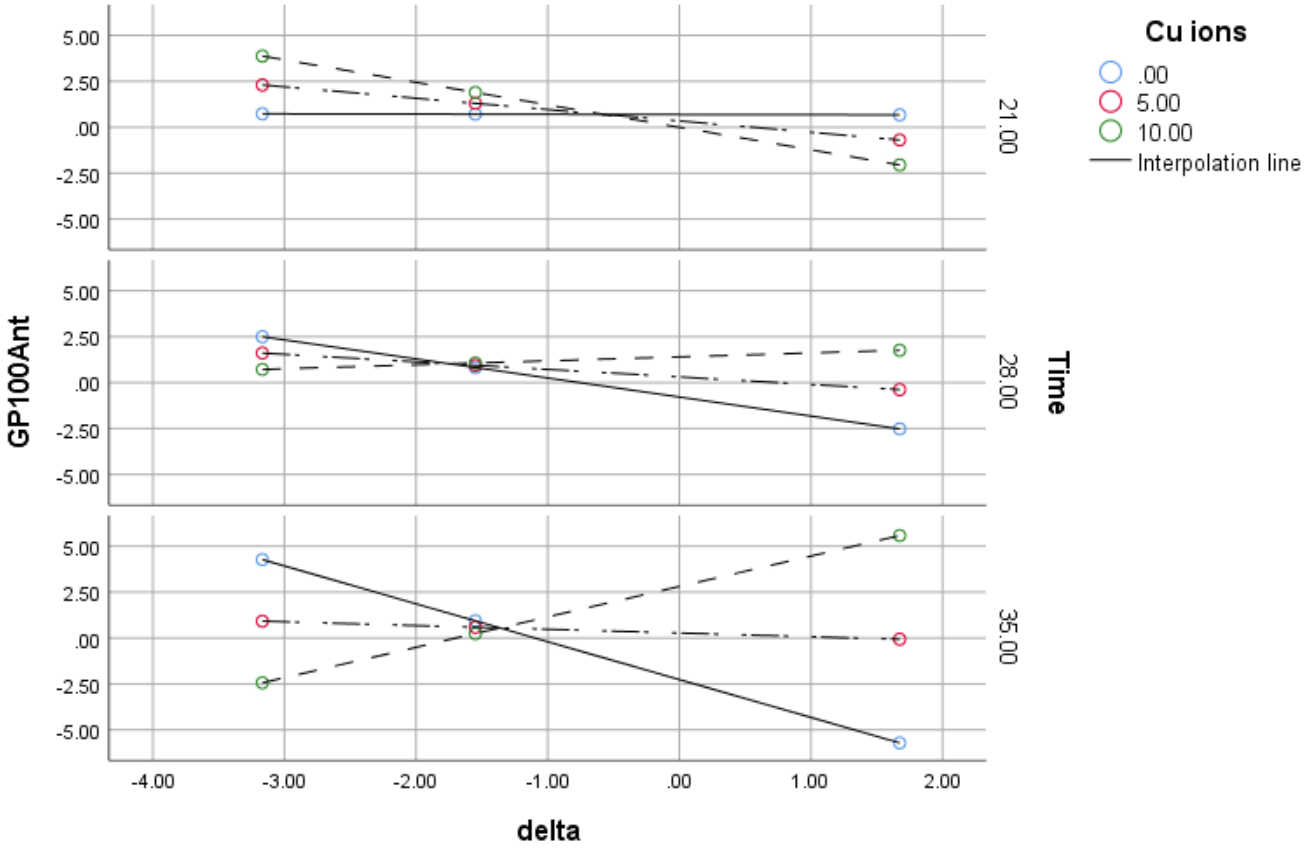

Figure 1

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

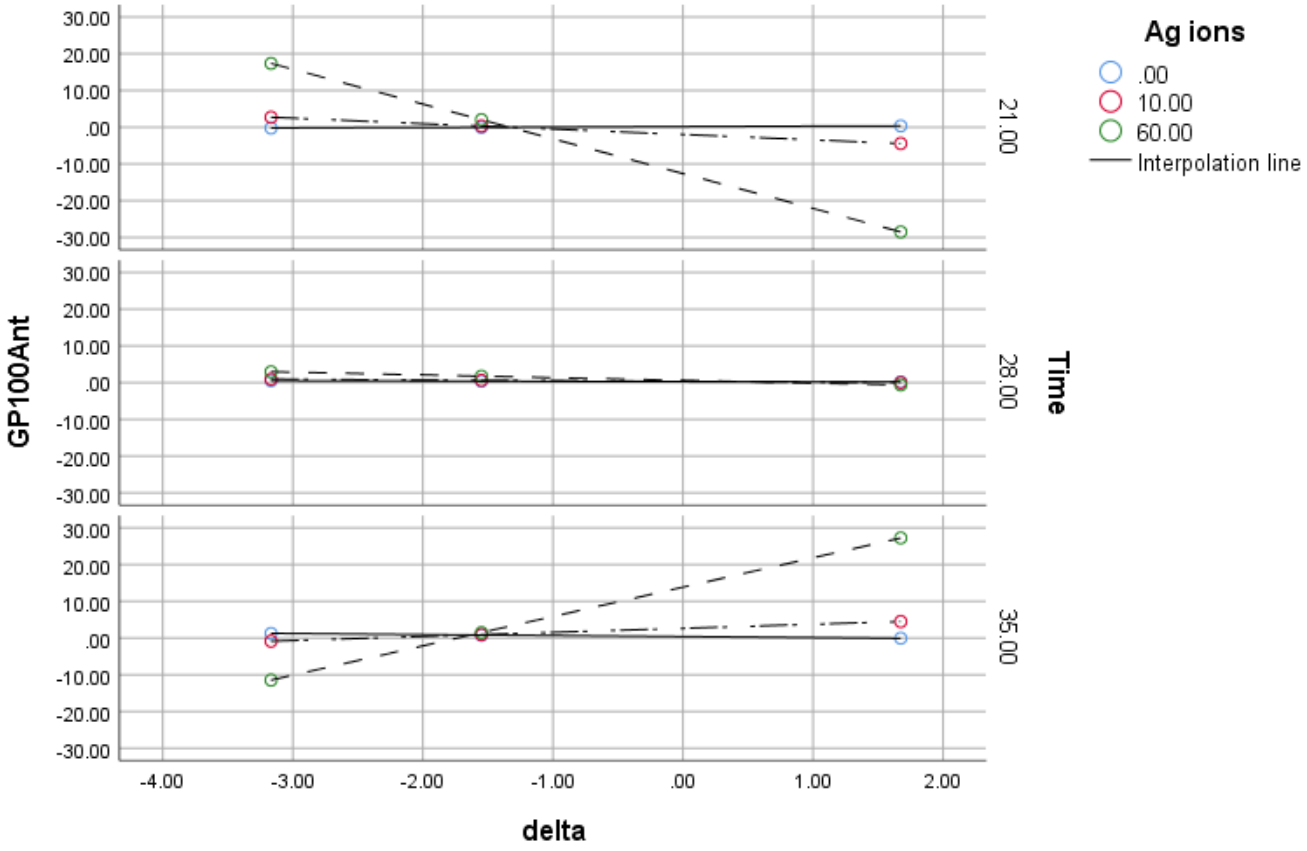

Figure 2

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

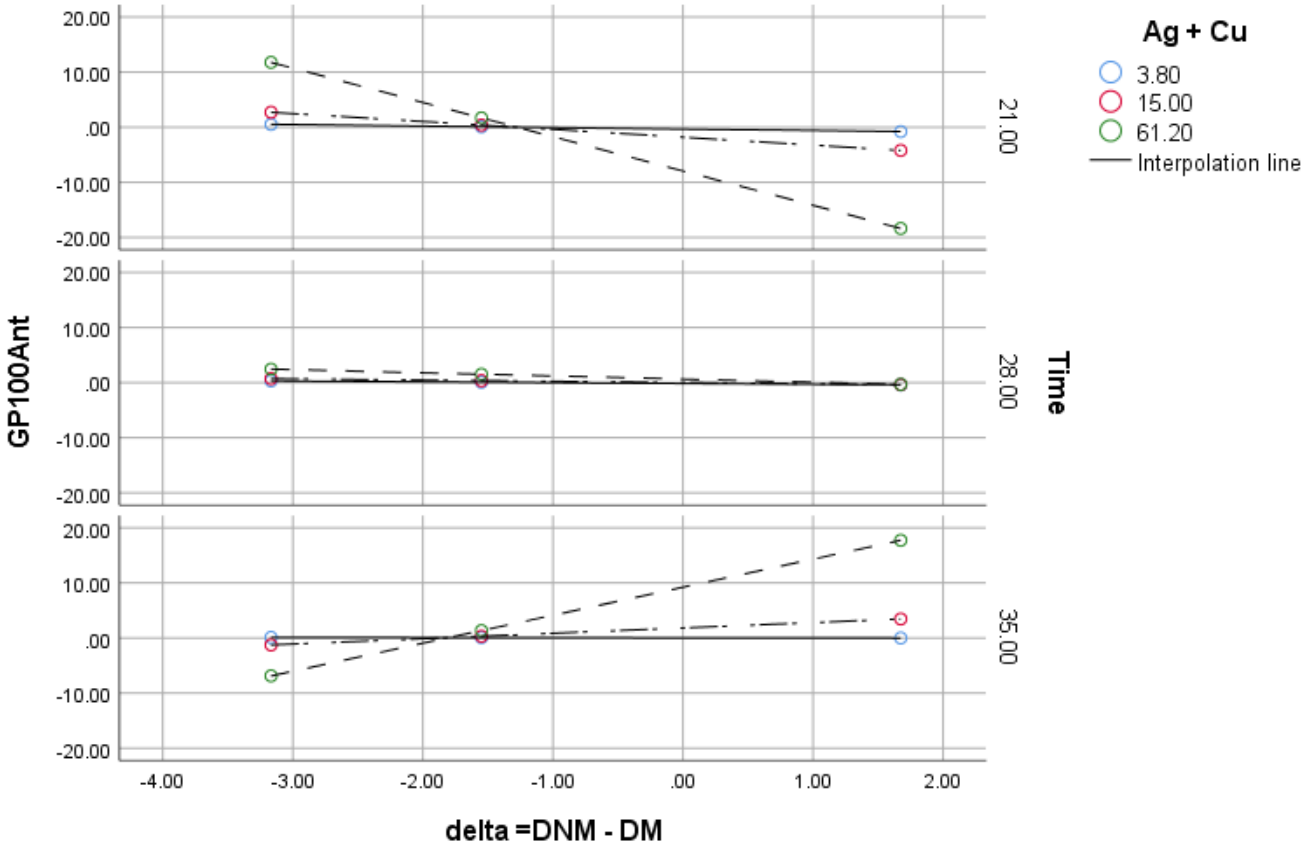

Figure 3

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

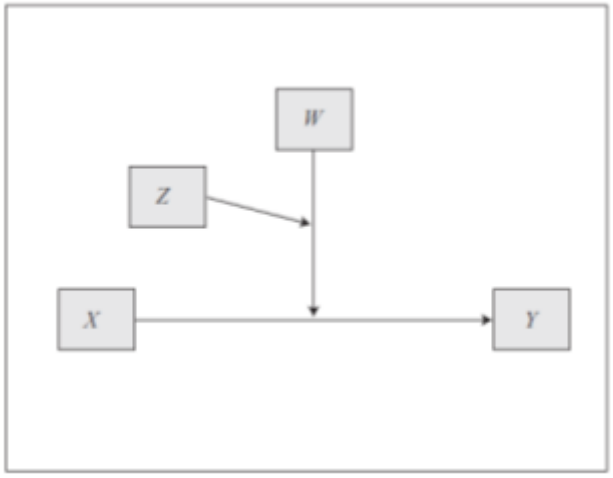

Figure 4

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

Supplementary Files

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