



Comparison of tissue culture-induced variation in triticale regenerants obtained by androgenesis and somatic embryogenesis

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Abstract

Triticale is becoming an increasingly important livestock crop production. This is evidenced by increasing triticale-producing areas and by improved yields. In addition, meeting the increasing demand for cereals involves the introduction of high-yielding and stress-resistant varieties into breeding. In vitro culture techniques can accelerate the development of new varieties. Therefore, it seems extremely important to develop efficient plant regeneration methods through in vitro cultures and to understand the mechanisms involved in gaining regenerants. Obtaining regenerants of triticale through somatic embryogenesis and androgenesis may lead to tissue culture-induced variation. In the present study, we compared regenerants obtained in both regeneration systems (anther and immature zygotic embryo cultures), considering the level of genetic and epigenetic changes observed in different DNA sequence contexts for methylated cytosine (CG, CHG, CHH). The changes concerning the DNA sequence (so-called sequence variation) and the changes concerning the DNA methylation patterns, i.e., the removal of methylated cytosine (DNA demethylation) and the introduction of methylation to cytosine (de novo DNA methylation), were analyzed. We observed that regenerants derived via somatic embryogenesis and androgenesis differ notably for demethylation in the symmetrical CG sequence context and de novo methylation in the asymmetrical CHH context. These changes may be related to the reprogramming of microspore development from gametophytic to sporophytic and lack of such process in zygotic embryos.

Keywords Triticale · Somatic embryogenesis · Androgenesis · Tissue culture-induced variation · metAFLP · DNA methylation

Introduction

The botanical tribe *Triticeae* includes cereal genera such as *Triticum*, *Hordeum*, *Secale*, *Aegilops*, and others (Soreng et al. 2017). Species belonging to different genera of this tribe can cross to a greater or lesser extent. Thanks to such abilities, the cross between wheat and rye, *Triticosecale* was created by Wilson in 1875. Wilhelm Rimpau evaluated the first fertile triticale plants in 1888 (Franke and Meinel 1990). After more than 130 years, triticale has developed from a botanical peculiarity to an economically important cereal, as shown by the increasing yield of this cereal from 2.5 t ha⁻¹

in 1975 to 4 t ha⁻¹ in 2020 (FAO 2020). By crossing wheat with rye, it has become to combine the advantages of wheat, such as high protein content in the grain, with the disease, cold, and drought resistance of rye. It is still essential to understand the biology of this species through biochemical, biological, and genetic studies, in which in vitro technique cultures can be a helpful tool.

Somatic embryogenesis is crucial for cereal transformation processes since somatic embryos serve as an ideal material for genetic transformation studies because of their competency in expressing incorporated DNA. However, an efficient and effective regeneration system is necessary for applying genetic transformation and functional genomics studies of important agronomy plants. Somatic embryogenesis in triticale was first reported at the end of 1980s (Stolarz and Lörz 1986; Zimny and Rybczyński 1986). In those studies, somatic embryos were obtained from triticale callus. Since triticale is a new, intergeneric hybrid, there is a constant need to expand the genetic diversity and the number

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of varieties for breeding. In the aspect of deriving new triticales varieties, in vitro culture methods can be extremely useful. In this context, cultures of isolated microspores or anther cultures to obtain doubled haploid (DH) plants play a significant role. For triticales, two systems were known for the production of DH plants, the first based on chromosome elimination (Laurie and Bennett 1989) and the second based on microspore embryogenesis (androgenesis) in anther culture or isolated microspore cultures. First triticales anther culture-derived haploid regenerants were obtained by Wang et al. (1973). In contrast, work on protocols for obtaining doubled triticales haploids in isolated microspore cultures has been successful for the group managed by Pauk et al. (2000). Subsequent years of research developed existing protocols for obtaining triticales DH to improve regeneration efficiency, but a significant breakthrough was using a spikes cooling step (Charmet and Bernard 1984; Lantos et al. 2014; Wurschum et al. 2012). Cold stress is a major factor that alters microspore development from the gametophytic to the sporophytic pathway (Smýkal 2000; Testillano 2020). Finally, the development of in vitro cultures based on androgenesis in triticales resulted in the first DH plants for this species (Fossati et al. 1998; González et al. 1997; Wędzony 2003).

Obtaining regenerants of triticales either by somatic embryogenesis or androgenesis is associated with somaclonal or otherwise tissue culture-induced variation (TCIV). Plants obtained in artificial conditions show some genetic (Bebeli et al. 1988) or biochemical changes (Deepthi 2018), which may be reflected by plant morphology (Jordan and Larter 1985), DNA sequence (Machczyńska et al. 2015), DNA (Machczyńska et al. 2014a) or histone (Pérez et al. 2015) methylation patterns, or gene transcription level (Zhang et al. 2010). Since triticales regenerants can be used for a variety of purposes, it is vital whether such plants will be loaded with TCIV or not. While for the improvement of genetic diversity, the presence of somaclones is positive (Jain 2001; Schellenbaum et al. 2008), for the production of highly genetically uniform material, such variability can be a problem because it leads to a loss of reliability (Rani and Raina 2000). Furthermore, a disadvantage of using somaclonal variation as a source of somaclones is that the results of somaclonal variation cannot be predicted. Also, it can be challenging to control changes of a genetic or epigenetic nature in regenerants.

Nevertheless, recent studies on barley obtained in immature zygotic embryos and anther cultures have shown that TCIV levels can be influenced by supplementing induction media with simple micronutrients such as silver and copper salts (Bednarek and Orłowska 2020a; Orłowska et al. 2021). In the case of triticales, no such extensive studies on in vitro induced variation have been conducted so far, like in older and naturally derived species. Considering the above and that triticales is an evolutionarily new species, artificially

created and characterized by genetic instability, studies on TCIV may be necessary as basic research and a practical one for obtaining triticales regenerants in tissue cultures.

The present work aimed to compare qualitative and quantitative characteristics of metAFLP for triticales regenerants obtained via androgenesis and somatic embryogenesis. In addition, this work attempts to indicate which way of obtaining triticales regenerants may be more beneficial regarding the tissue culture-induced variation in regenerants.

Materials and methods

Plant material

Triticales donor plants (*X Triticosecale* Wittm., cultivar T28/2) were grown under controlled conditions to an appropriate developmental stage from which explants could be collected. The obtained plants came from a cross between cv. Presto × cv. Mungis and made available by Sylwia Oleszczuk (Plant Breeding and Acclimatization Institute-NRI, Radzików, Poland). Tillers for anther culture were cut when microspores were at the mid-to the uninucleate stage. Then, the cut tillers were subjected to cold stress (4 °C) in the dark for 20 days. After this time, the spikes were sterilized, and anthers were removed onto induction media. The collection of tillers for immature zygotic embryos (IZE) culture was after 12–16 days of self-pollination. Caryopses were immediately removed from the harvested spikes, and after disinfection, IZE were excised out and lined on induction media (IM).

Callus induction and plant regeneration

Anther cultures (androgenesis)

In anther cultures, 190-2 medium (Zhuang and Xu 1983) with 90 g l⁻¹ maltose and 438 mg l⁻¹ glutamine, supplemented with 2 mg l⁻¹ 2,4-dichlorophenoxyacetic acid and 0.5 mg l⁻¹ kinetin, was used for induction. The anthers were incubated in the dark at 26 °C. Induction media were made in nine T1-T9 variants testing the effects of copper and silver ions, and time on genetic and epigenetic variation induced in plant tissue culture. Variant T1 contained a standard amount of CuSO₄ × 5H₂O (0.025 mg l⁻¹) and was not supplemented with silver salts (AgNO₃). In contrast, the final concentrations in variants T2–T9 ranged from 0.1 to 10 μM CuSO₄ × 5H₂O and from 0 to 60 μM AgNO₃. The incubation time ranged from 35 to 49 days. From the 35th day of incubation, emerging calli and embryos were transferred to regeneration medium 190-2 (Zhuang and Xu 1983) supplemented with naphthalene acetic acid at a concentration of 0.5 mg l⁻¹ and kinetin at a concentration of 1.5 mg l⁻¹.

Incubation on regeneration medium was carried out at 26 °C in a 16 h/8 h light/dark photoperiod. Green plants were rooted in glass flasks with N6I medium (Chu 1978) supplemented with indole-3-acetic acid at a dose of 2 mg l⁻¹ and then transplanted into pots with a mixture of soil and sand and vernalized for six weeks at 4 °C. Leaves were collected from young plantlets for DNA isolation.

Immature zygotic embryo culture (somatic embryogenesis)

In immature zygotic embryo cultures, MS medium (Murashige and Skoog 1962) with 2 mg l⁻¹ of 2,4-dichlorophenoxyacetic acid was used to induce somatic embryogenesis. As in the anther cultures, induction media were prepared in nine variants with the same Cu and Ag ion supplementation and identical incubation time of the explants on IM. IZE were incubated on IM at 26 °C in a photoperiod of 16 h/8 h (light/dark). After a few days, the first callus and embryos were recorded and transferred to a regeneration medium the same for anther culture. All subsequent steps of regeneration and obtaining plantlets were performed in the same way as for anther cultures. From the seedlings of regenerants, leaves were taken for DNA isolation. Finally, regenerants that were obtained after incubation of explants on induction medium after 35, 42, and 49 days were taken for analysis.

Molecular assays

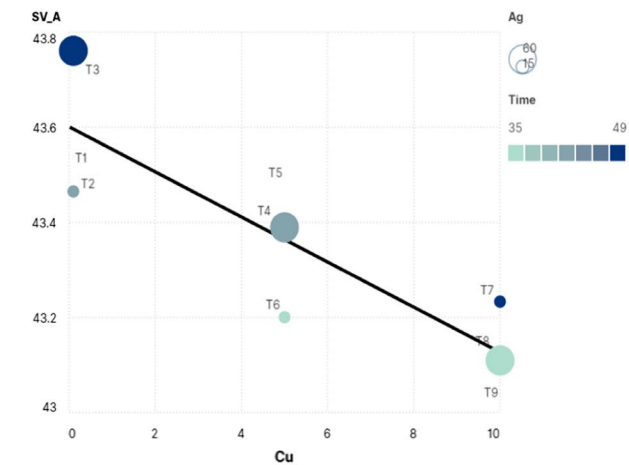
Young leaves of regenerants from anther cultures, IZE cultures, and donor plants were collected. DNA was extracted using the Plant DNeasy MiniPrep Kit (Qiagen, Hilding, Germany). DNA concentration was checked using a Nanodrop spectrophotometer (Thermo Scientific). The integrity of DNA was tested in 1.2% agarose gel electrophoresis. For methylation sensitive Amplified Fragment Length Polymorphism (metAFLP) manipulation, there were prepared 2 × 500 ng of DNA for each plant.

Forty-four regenerants derived from anther cultures and a donor plant and 45 regenerants derived from IZE cultures and the donor plant were selected for analysis by the metAFLP technique. All steps of the metAFLP technique were performed with the previously published procedure of Bednarek et al. (2007) with modifications according to Machczyńska et al. (2014b). DNA fragments were amplified in two steps: a preselective PCR reaction followed by selective PCR with appropriate primers (Pachota et al. 2022). After selective PCR, DNA samples were diluted with water 1:20. PCR products were electrophoresed in 7% polyacrylamide gel and fixed on X-ray films, and then visualized via developing photography chemicals. The metAFLP products (clearly identifiable and repetitive) were scored as present (1) or absent (0) based on the electropherograms. There were two sets of bands profiles, one generated from the separation

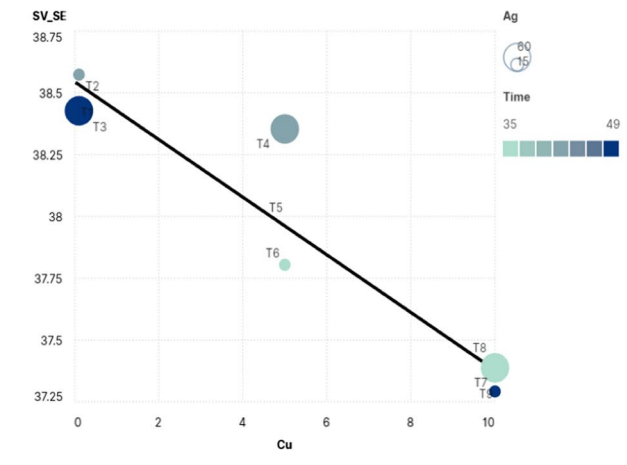
of DNA fragments derived from the Acc65I and MseI, and second generated with KpnI and MseI restriction enzymes. The DNA strand arrays obtained were matched against each other according to the primers used for the selective PCR reaction, and the presence of DNA fragments was compared. The Acc65I and MseI enzyme pair allow information to be obtained regarding changes in sequence and DNA methylation. In contrast, KpnI and MseI enzymes cut the same DNA samples to obtain information regarding changes in the DNA sequence. Such a comparison of the two DNA fragment matrices is possible because the properties of the restriction enzymes used, Acc65I and KpnI, are isoschizomers that recognize the same nucleotide sequence but cut DNA depending on the presence of a methyl group in the cytosine. Appropriately selected plant material consisting of a donor plant that is the generative progeny of regenerants obtained by androgenesis and the regenerants derived from it provide a basis for estimating tissue culture-induced variation (TCIV). Hence, comparing with each other the DNA profiles for the donor plant and its regenerants obtained from DNA cutting with two sets of restriction enzymes allowed us to reveal changes in DNA sequence (sequence variation—SV) and changes in DNA methylation (DNA demethylation—DMV) and de novo DNA methylation—DNMV). Using specially designed selective primers directed toward symmetric and asymmetric DNA sequences evaluated in the metAFLP technique allows identifying CG, CHG, and CHH methylation changes contexts. A detailed description of the metAFLP enumeration system and the assignment of methylation contexts was reported previously (Orłowska and Bednarek 2020). For individual regenerants obtained in IZE cultures and anther cultures, the level of metAFLP characteristics (SV, DMV, DNM) forming TCIV was quantified and expressed as percentages (%). Then, the characteristics for the regenerants from IZE and anther cultures were compared on graphs, also taking into account the methylation contexts (CG, CHG, and CHH). The graphs were generated using SCImago Graphica Beta 1.0.17 software (www.graphica.app).

Results

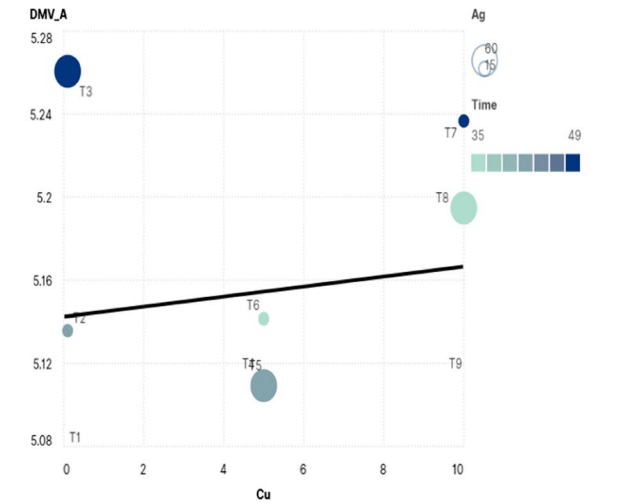
Only minor differences could be detected when comparing regenerants derived from androgenesis ‘A’ and somatic embryogenesis ‘SE’ using general metAFLP characteristics concerning Cu(II) ion concentration in the induction media (IM). In both cases, the increase in Cu(II) concentration results in the decrease of sequence variation (SV) (Fig. 1 a, b). No apparent dependence on the time of anther culture is seen. The same is valid for the Ag(I) concentration. DNA demethylation (DMV) increases in ‘A’ and ‘SE’ with Cu(II) elevation in the induction medium (Fig. 1 c, d). However,



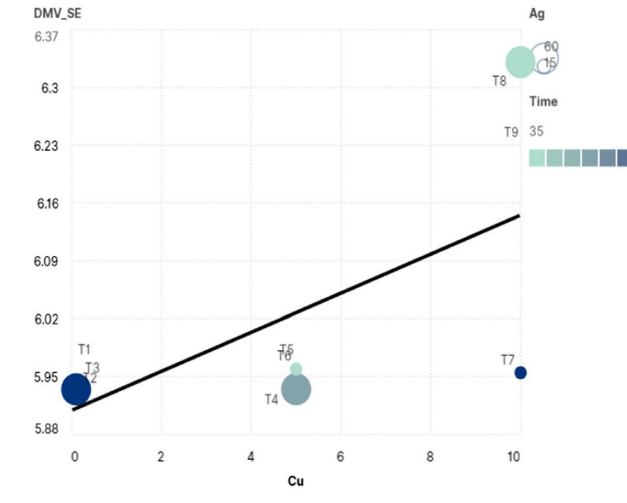
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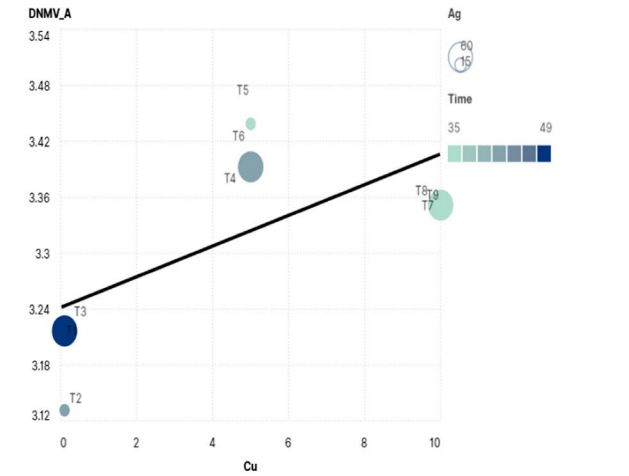
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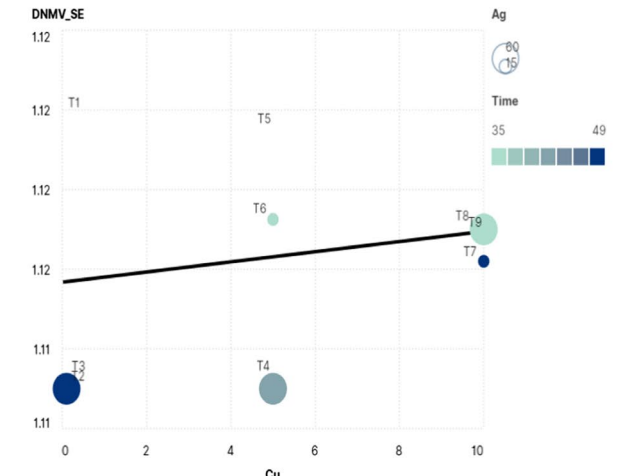
c



d



e



f

Fig. 1 The relationship between Cu(II) and sequence variation (SV) for anther-derived ‘A’ regenerants (a) and somatic embryo-derived regenerants ‘SE’ (b); Cu(II) and DNA demethylation (DMV) for ‘A’ (c), and ‘SE’ (d); Cu (II) and de novo DNA methylation (DNMV) for ‘A’ (e) and ‘SE’ (f) based on general metAFLP characteristics. Time (days) of tissue cultures is given as color; Ag (I) concentration is shown as circles of different diameters

the trend is more pronounced in ‘SE.’ The DMV is more evident under the highest Ag(I) concentration (T8), whereas under the lowest one and the longest time of anther culture is the lowest. Under anther culture, the reverse is observed. De novo DNA methylation (DNMV) is higher in ‘A’ than in ‘SE’ when Cu(II) increases (Fig. 1e, f). Both time and Ag(I) are not key players here.

The relationship between DNMV and SV evidences that with the increase of DNMV, SV decreases (Fig. 2a, b). However, no apparent dependence is seen on Ag(I) concentration and time of anther culture. A similar comparison of DMV and SV shows that SV slightly increases in ‘A’ but decreases in ‘SE’ (Fig. 2c, d). Furthermore, the highest DMV is reflected by trials T8 and T9 with the highest Cu(II) and Ag(I) in ‘SE’, whereas in ‘A’ also, high Cu(II) and Ag(I) is observed with increased DMV; however, the dependence is less pronounced.

Comparison of sequence variation for regenerants derived from androgenesis ‘A’ and somatic embryogenesis ‘SE’ in sequence contexts shows that asymmetric CHH (Fig. 3a) and symmetric CG (Fig. 3b) sequences seem to be nearly

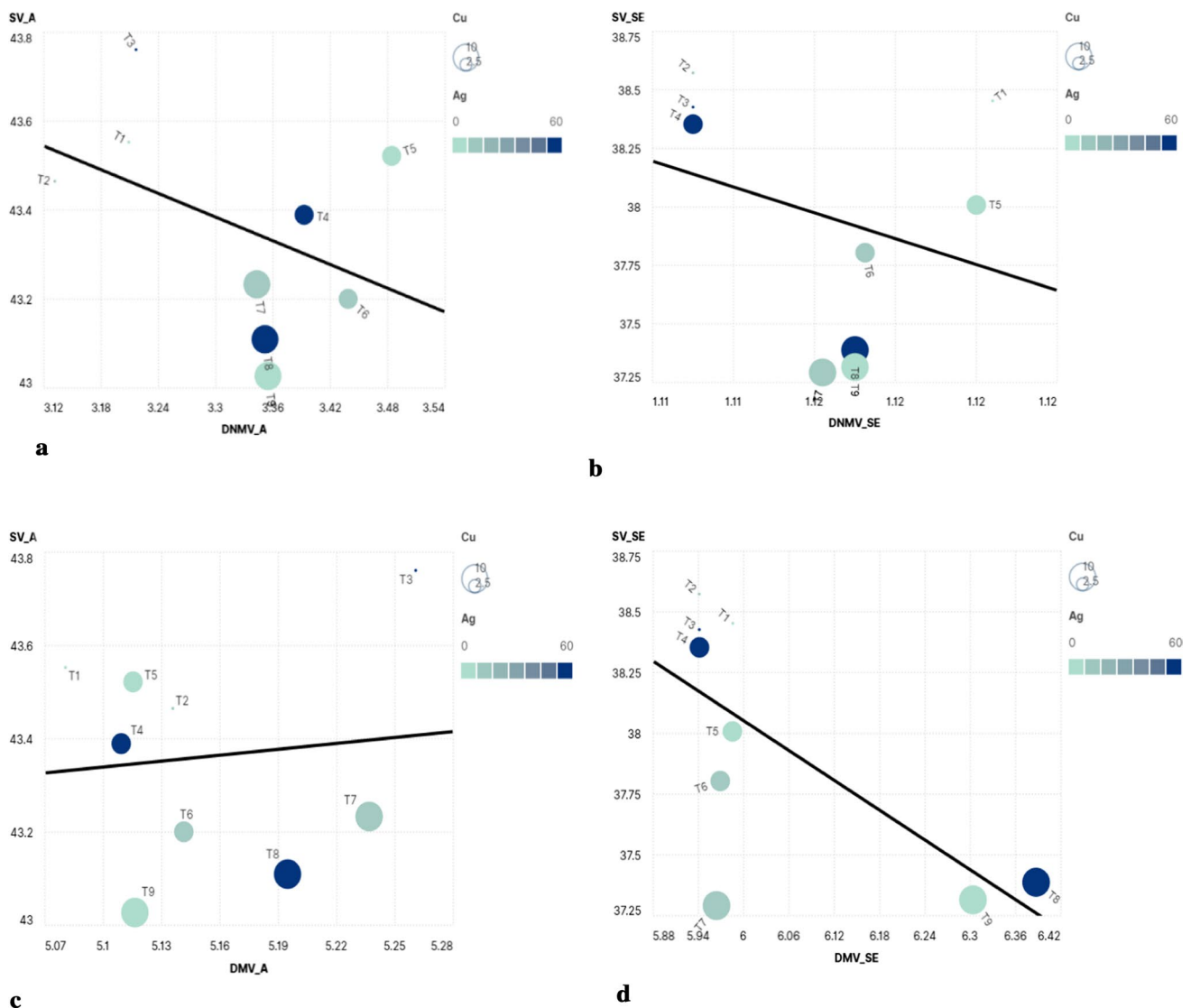


Fig. 2 The relationship between sequence variation (SV) and de novo DNA demethylation (DNMV) for anther-derived ‘A’ regenerants (a) and somatic embryo-derived regenerants ‘SE’ (b) and between

SV and demethylation (DMV) ‘A’ (c), and ‘SE’ (d) based on general metAFLP characteristics; Ag(I) concentration is given as color; Cu(II) concentration is shown as circles of different diameters

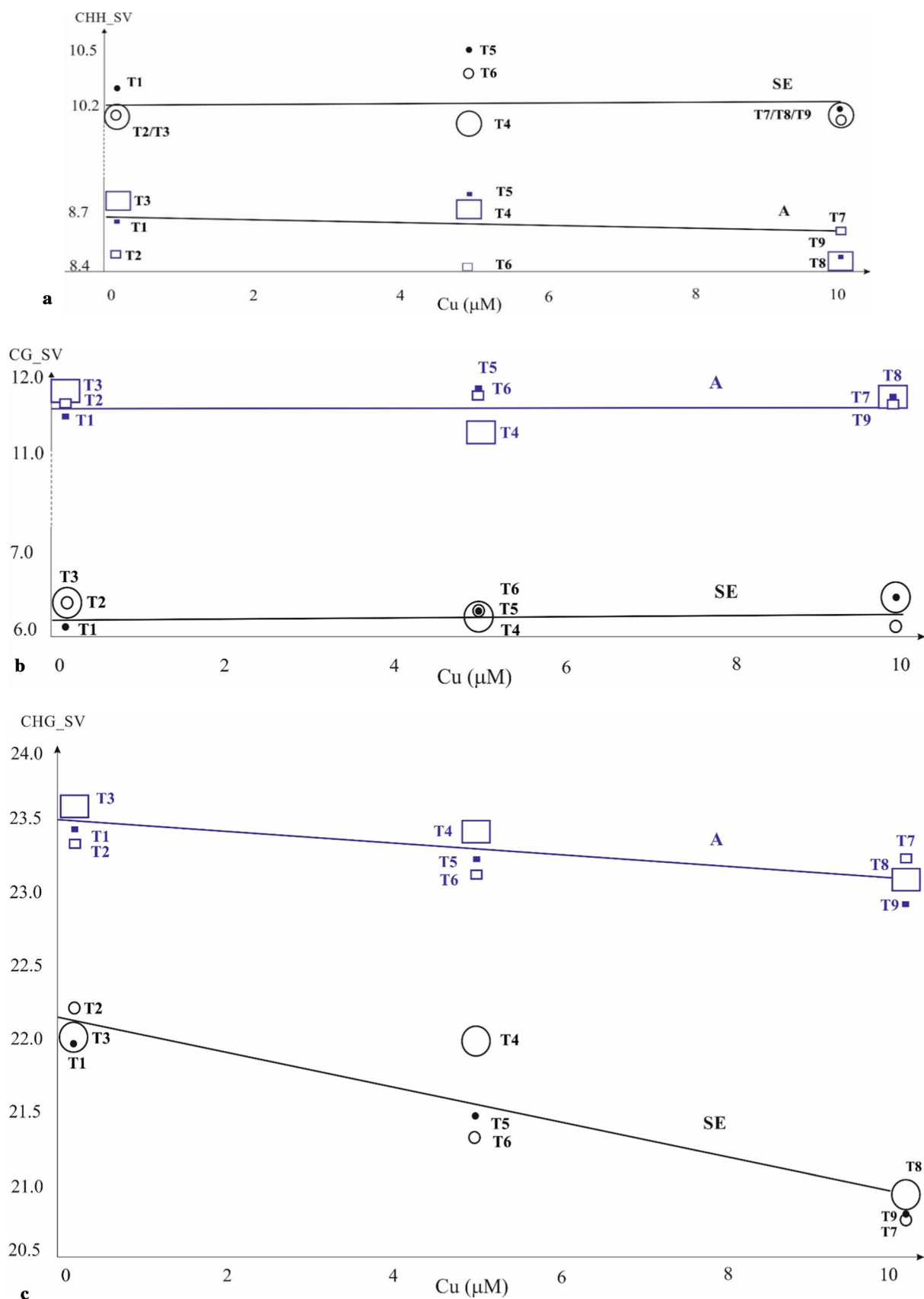


Fig. 3 **a** The relationship between Cu(II) ion concentration in the IM and sequence variation level (in %) affecting CHH asymmetric sequence context for anther-derived ‘A’ regenerants and somatic embryo-derived regenerants ‘SE’; Ag(I) concentration is given as circles/dots and squares/filled squares from the smallest to the biggest (0, 10, 60 μ M). **b** The relationship between Cu(II) ion concentration in the IM and sequence variation level (in %) affecting CG symmetric sequence context for anther-derived ‘A’ regenerants and somatic embryo-derived regenerants ‘SE’; Ag(I) concentration is given as circles/dots and squares/filled squares from the smallest to the biggest (0, 10, 60 μ M). **c** The relationship between Cu(II) ion concentration in the IM and sequence variation level (in %) affecting CHG symmetric sequence context for anther-derived ‘A’ regenerants and somatic embryo-derived regenerants ‘SE’; Ag(I) concentration is given as circles/dots and squares/filled squares from the smallest to the biggest (0, 10, 60 μ M)

constant with increasing Cu(II) ion concentration in the IM (maybe in ‘A’ a slight decrease in sequence variation level is evidenced). However, SV affecting CHH contexts is higher for the ‘SE’ than for ‘A’. The opposite is observed for the CG sequence contexts. Contrasting ‘SE’ and ‘A’ for the CHG sequence contexts shows that increasing Cu(II) ions decreases sequence variation within the contexts in both tissue culture regeneration methods (Fig. 3c). However, the decrease in ‘SE’ is higher than in ‘A’ with increased Cu(II), but a higher level of SV is evidenced for ‘A’.

Comparing the contexts, the DNA demethylation level (DMV) for “SE” is higher than that for “A” in CHH and CHG contexts (Fig. 4a, c). In contrast, the opposite situation is observed in the CG context, where the DMV level is higher in “A” than in “SE” (Fig. 4b). The evaluation of the impact of Cu(II) ions in the IM on DNA demethylation level within sequence contexts for ‘SE’ and ‘A’ demonstrates that in the CHH context, DNA demethylation affecting ‘SE’ regenerants somewhat increases with Cu(II) concentration, whereas decreases for the ‘A’ derived plants (Fig. 4a). Comparing ‘SE’ and ‘A’ in CG context indicates the increase of SV with Cu(II) with higher changes affecting ‘SE’ than ‘A’ (Fig. 4b). Nevertheless, the level of DMV is higher for A than for ‘SE’. No change in DMV due to Cu(II) ion concentration was found for the CHG sequence context (Fig. 4c). Again, different levels of DMV could be seen for ‘SE’ and ‘A’ depending on sequence context type, which is higher for ‘SE’ in the CHH and CHG contexts. No evident dependence on Ag(I) of anther culture could be seen.

Possibly the most pronounced differences between ‘SE’ and ‘A’ derived regenerants could be seen in the case of relationships between Cu(II) and de novo DNA methylation (DNMV) affecting asymmetric (CHH) sequence contexts (Fig. 5a). While in ‘SE’, increasing Cu(II) seems not to impact DNMV in ‘A’ de novo methylation increases with Cu(II) concentration. Still, DNMV in ‘SE’ is higher than in ‘A’. The DNMV of other sequence contexts (Fig. 5b, c) for ‘SE’ and ‘A’ behave similarly and is constant. However,

DNMV of those contexts is higher for ‘A’ than for ‘SE’ derived regenerants. Again, no apparent influence of Ag(I) could be seen.

Discussion

The comparison of the androgenesis ‘A’ and somatic embryogenesis ‘SE’ due to change in Cu(II) concentration supplemented in the induction media (IM) indicates that, in general, DNA demethylation and de novo methylation differ between the two approaches. Such a trend aligns with the cell reprogramming step (Feng et al. 2010). In anther cultures, DNA demethylation accompanies the switch of microspores from gametophytic to sporophytic fate (Solís et al. 2012). In the case of embryogenesis, in light of the available studies, it appears that the initiation of embryogenesis, whether it occurs as zygotic embryogenesis or any asexual embryogenesis, including somatic embryogenesis, is associated with strong activation of DNA methyltransferases (Jullien et al. 2012), which is not dependent on fertilization (Park et al. 2020) and does not differ between zygotic and somatic embryogenesis. In contrast, the initiation of embryogenesis is not initiated by the fertilization process but by other signals that affect methylation patterns in the early embryo regardless of whether it originates from zygotic or asexual embryogenesis (Markulin et al. 2021). Apart from stress conditions, the synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D), is a factor that significantly influences the initiation of somatic embryogenesis (Ikeda-Iwai et al. 2002; Mordhorst et al. 1998). Furthermore, exogenous auxin, which promotes somatic embryogenesis, increases DNA methylation in carrot cell cultures (Lo Schiavo et al. 1989). Such effect is due to the 2,4-D regulation of genes encoding DNA methyltransferases and downregulation of demethylases (Grzybkowska et al. 2018). However, another work on somatic embryogenesis indicates an inverse effect of 2,4-D on methylation levels and embryogenic competence, as reflected by DNA hypomethylation at early embryonic stages in *Pinus nigra* (Noceda et al. 2009). Given the divergent results of global DNA methylation during somatic embryogenesis, it is assumed that the level of DNA methylation is not explicitly related to embryogenesis but is related to the effect of in vitro culture per se (composition of the media) and probably reflects the epigenetic state of the explants (Wójcikowska et al. 2020). In embryogenesis, resetting or decreasing DNA methylation levels could be an essential step of cell fate transition from the vegetative to embryogenic (Nic-Can and De la Peña 2014). In some way, DNA demethylation in ‘A’ and ‘SE’ plays the same role in both two approaches; however, in the case of microspores with haploid chromosomes, the reestablishment of the methylation pattern, especially in asymmetric sequence context

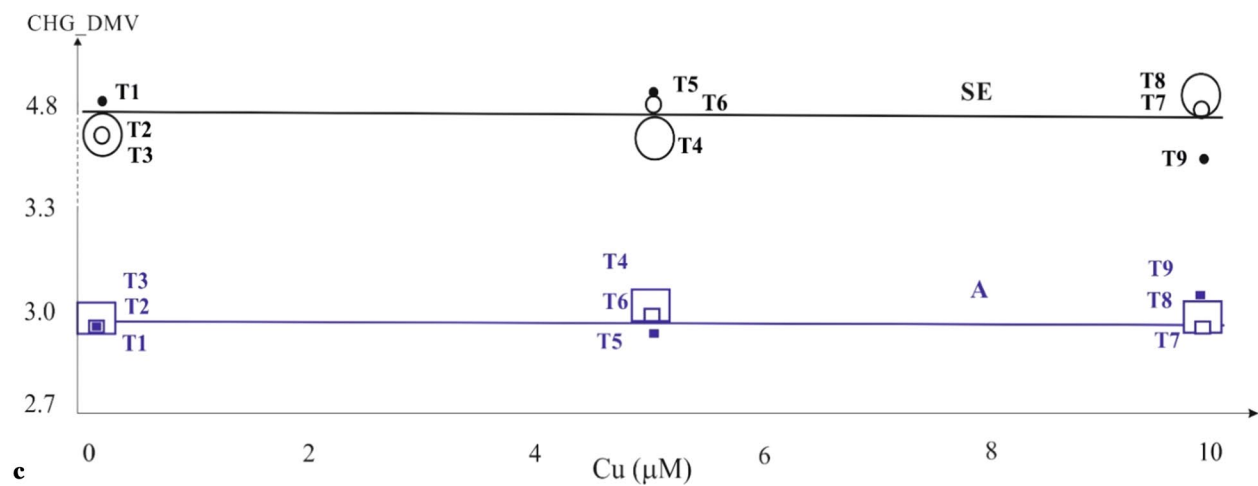
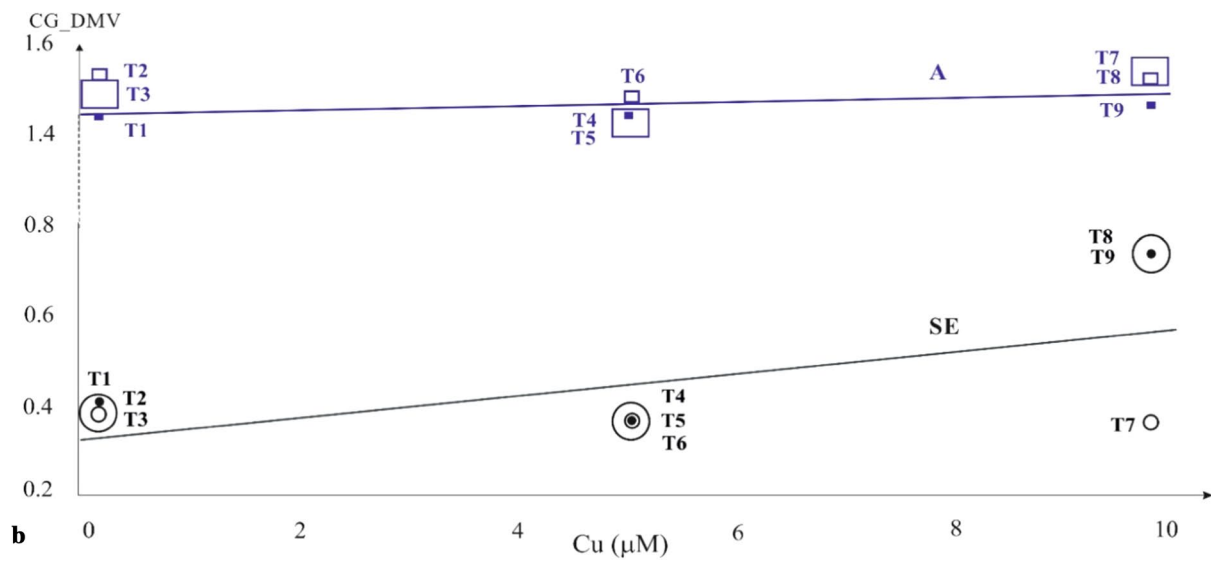
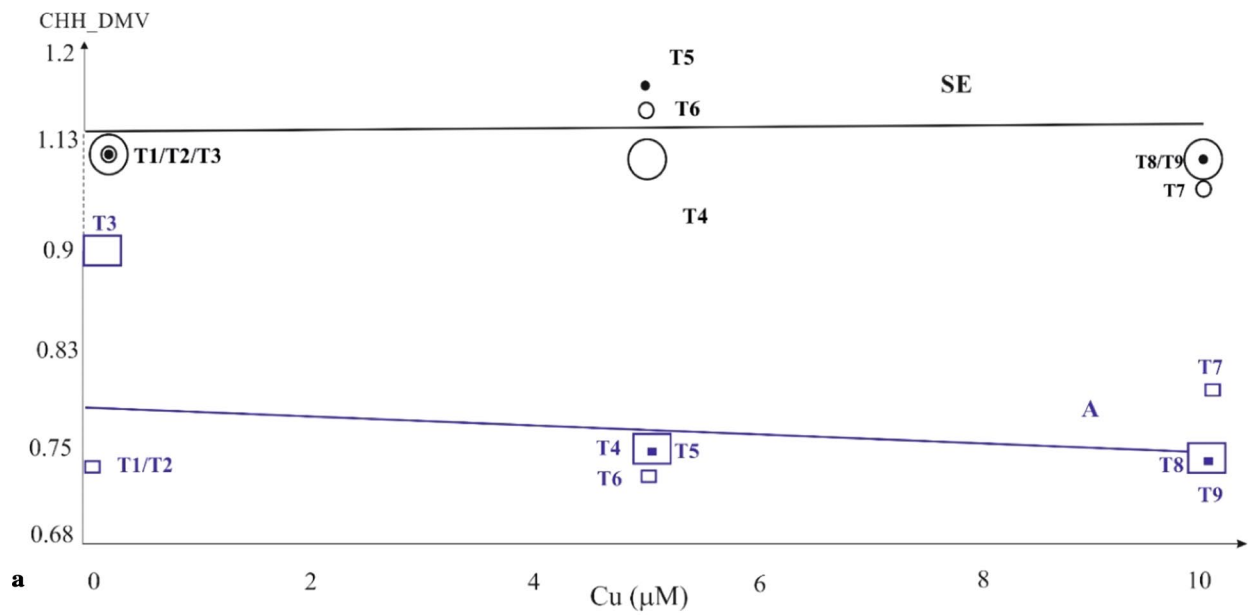


Fig. 4 **a** The relationship between Cu(II) ion concentration in the IM and DNA demethylation variation level (in %) affecting CHH asymmetric sequence context for anther-derived ‘A’ regenerants and somatic embryo-derived regenerants ‘SE’; Ag(I) concentration is given as circles/dots and squares/filled squares from the smallest to the biggest (0, 10, 60 μ M). **b** The relationship between Cu(II) ion concentration in the IM and DNA demethylation variation level (in %) affecting CG symmetric sequence context for anther-derived ‘A’ regenerants and somatic embryo-derived regenerants ‘SE’; Ag(I) concentration is given as circles/dots and squares/filled squares from the smallest to the biggest (0, 10, 60 μ M). **c** The relationship between Cu(II) ion concentration in the IM and DNA demethylation variation level (in %) affecting CHG symmetric sequence context for anther-derived ‘A’ regenerants and somatic embryo-derived regenerants ‘SE’; Ag(I) concentration is given as circles/dots and squares/filled squares from the smallest to the biggest (0, 10, 60 μ M)

and the symmetric one (CHG) being either under epigenetic or partially epigenetic control may be problematic. That is possibly why DMV in ‘SE’ is more apparent than in ‘A’, whereas DNMT in ‘A’ is more apparent than in ‘SE’.

The analysis of SV as a function of Cu(II) affecting different contexts shows that in CG and CHH ones, the difference between ‘A’ and ‘SE’ is only quantitative, whereas CHG-related SV drops faster in ‘SE’ than in ‘A’. In androgenesis, CHG sequence context methylation is controlled (epi)genetically, whereas CHH is via an epigenetic mechanism (Gent et al. 2013; Law and Jacobsen 2010; Stroud et al. 2014; Zemach et al. 2013). Assuming that epigenetics participates in both cases and methylation leads to SV, it is apparent that putative problems with DNA methylation reestablishment could explain a slight decrease in SV for CHH in ‘A’.

DMV due to Cu(II) in the IM is most pronounced for the CG context. While in ‘A’, the increase in DMV of the contexts is less evident with Cu(II) in the IM, in SE, it seems to proceed faster; however, in general, it is less quantitative than in ‘A’. Most probably, DNA demethylation of the CG site reflects the process of DNA demethylation during the replication stage (Liu and Lang 2020). The differences in DMV of the CG contexts concerning ‘A’ and ‘SE’ may reflect the differences in DNA demethylation of the two sources of explants. It cannot be excluded that the metAFLP approach could detect more changes in putatively more heterozygous explants that originated from diploid tissue, which might result in overall lower values of DMV in ‘SE’. In CHH and CHG sites, DMV behaves nearly identically in ‘A’ and ‘SE’, with a lack of decrease or increase of its values for the CHG in ‘A’ and ‘SE’ and negligible decrease in DMV in ‘A’ for CHH context. Interestingly, the overall value of DMV for all Cu(II) concentrations was lower for ‘A’ than for ‘SE’ for the CHG and CHH contexts. Possibly, the observed data reflect some epigenetic mechanisms affecting methylation of the two contexts in contrast to the CG one.

Here, Cu(II) concentration impacts CHH DNMT; with increased Cu(II) concentration, DNMT increases in ‘A,’

whereas ‘SE’ remains constant. Thus, DNMT of the CHH is under epigenetic control (Gent et al. 2013; Law and Jacobsen 2010; Stroud et al. 2014; Zemach et al. 2013). Changing the development pathway of microspores toward androgenesis requires epigenetic reprogramming occurring after induction of microspores into a totipotent state and initiation of microspore embryogenesis (androgenesis). Such reprogramming involves a global decrease in DNA methylation and activation of cell proliferation, followed by an increase in DNA methylation and differentiation into the embryo (El-Tantawy et al. 2014; Rodríguez-Sanz et al. 2014; Solís et al. 2012, 2015; Testillano et al. 2013). More detailed studies on specific DNA methylation contexts performed during somatic embryogenesis in soybean show that DNA methylation increases during somatic embryo development. To the contrary, the increase in methylation in the CHH context may be related to an active transcriptional silencing program that enhances genome-wide DNA methylation during somatic embryo development (Ji et al. 2019). In addition, it should be noted that asymmetric DNA methylation (CHH) is mainly removed from male gametes and then restored after fertilization. Additionally, CHH methylation levels are reduced in the nucleus of the sperm cell while showing significantly higher levels in microspores. It may suggest a conflict between methylation maintenance and demethylation during male germline development (Walker et al. 2018). Our result indicating higher DNMT levels with an increase in copper ions during ‘A’ compared to ‘SE’ may indicate the involvement of methylation in CHH context during reprogramming of microspore development and the absence of such an effect during somatic embryo formation in immature zygotic embryo cultures. Furthermore, CHH context DNA de novo methylation may be critical for the expression of genes impacting plant regeneration. Interestingly, CG and CHG DNMT was not affected by Cu(II) concentrations remaining constant. However, in both cases DNMT was higher for CG and CHG in ‘A’ than in ‘SE’ which is the reversed case compared to CHH DNMT. We tend to think that the differences reflect epigenetic aspects of DNMT affecting asymmetric sequence context.

The other intriguing result is that sequence variation decreases with DNMT in ‘SE’ while reversed, but a less expressed trend is seen in ‘A’. The result seems to be in line with the fact that the less DNA is methylated, the less it is subjected to mutations due to, i.e., oxidative stress (Lewandowska-Gnatowska et al. 2014). On the other hand, DNMT in ‘A’ and ‘SE’ behave in a very similar way. If oxidative stress is responsible for SV, one might expect that increasing DNMT the SV should increase. Still, the opposite is seen. While the explanation is not apparent, we speculate that maybe after the demethylation stage, de novo methylation

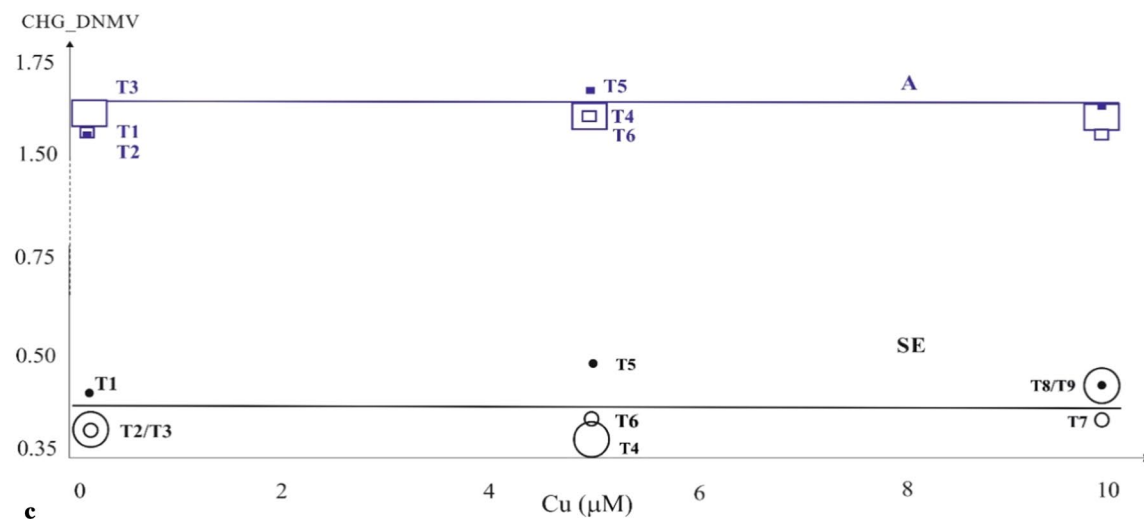
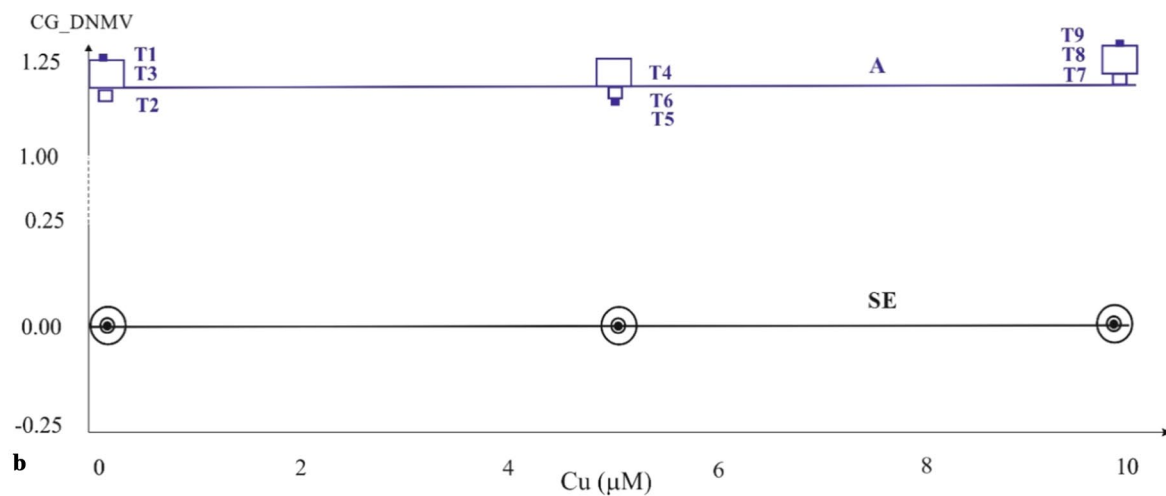
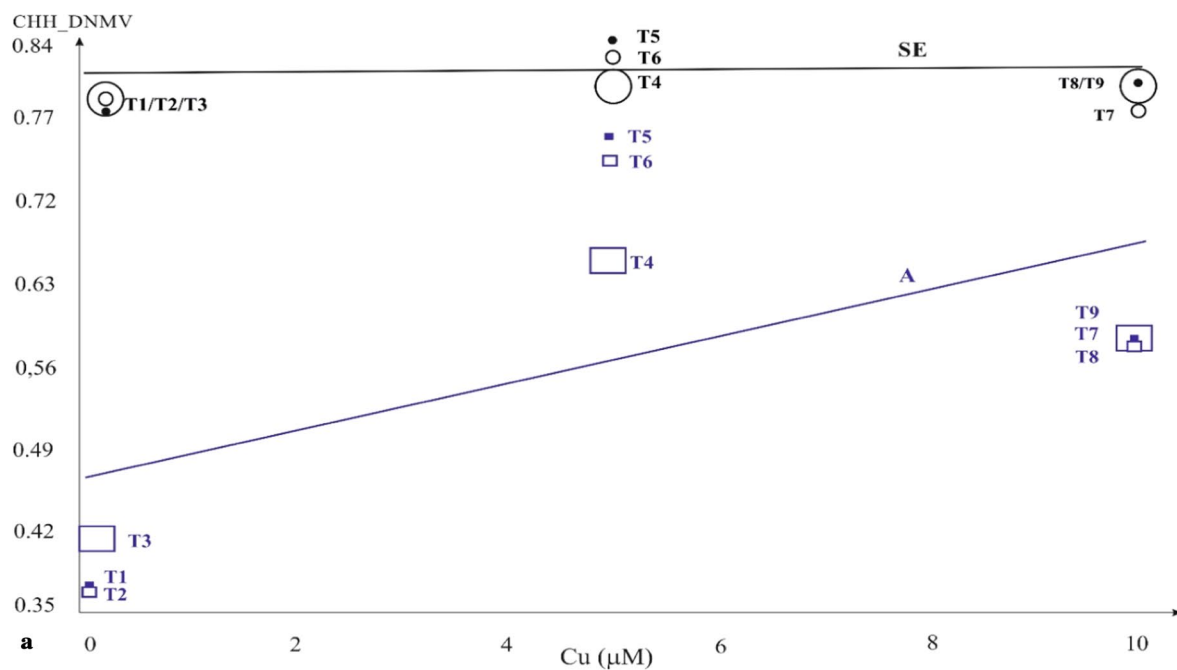


Fig. 5 **a** The relationship between Cu(II) ion concentration in the IM and de novo DNA methylation variation level (in %) affecting CHH asymmetric sequence context for anther-derived 'A' regenerants and somatic embryo-derived regenerants 'SE'; Ag(I) concentration is given as circles/dots and squares/filled squares from the smallest to the biggest (0, 10, 60 μ M). **b** The relationship between Cu(II) ion concentration in the IM and de novo DNA methylation variation level (in %) affecting CG symmetric sequence context for anther-derived 'A' regenerants and somatic embryo-derived regenerants 'SE'; Ag(I) concentration is given as circles/dots and squares/filled squares from the smallest to the biggest (0, 10, 60 μ M). **c** The relationship between Cu(II) ion concentration in the IM and de novo DNA methylation variation level (in %) affecting CHG symmetric sequence context for anther-derived 'A' regenerants and somatic embryo-derived regenerants 'SE'; Ag(I) concentration is given as circles/dots and squares/filled squares from the smallest to the biggest (0, 10, 60 μ M)

runs with limited stress conditions resulting in decreased SV with increased DNMTV.

Literature reports indicate a positive effect of silver ions on the efficiency of somatic embryogenesis (Bashir et al. 2022; Malik et al. 2021). Silver ions may also affect sequence variation (Ag^+ ions acted as a mediator), observed in barley regenerants obtained in anther cultures (Bednarek and Orłowska 2020a). Also, manipulating AgNO_3 concentration with prolonged culture time affected the production of green barley regenerants (Bednarek and Orłowska 2020b). Unfortunately, such an effect of silver ions was not observed in the case of triticale regenerants obtained by androgenesis and somatic embryogenesis based on the presented results. The effect of culture time was also not observed in the case of the experiment presented; therefore, this factor was not included in the graphs. In the present work, the culture time is related to incubating explants (anthers and immature zygotic embryos) on induction media. On the other hand, there are known reports where culture time/culture duration influenced the production of secondary metabolites and biomass in plant tissue cultures (de Andrade Figueiró et al. 2010). Furthermore, the manipulation of plant tissue culture duration is reflected in somaclonal variation, which increases with a multiplying number of subcultures (passages) and may relate to accumulating changes in DNA methylation (Eeuwens et al. 2002; Rival et al. 2013).

Summing up, androgenesis and embryogenesis differ in sequence contexts, with the most exhibited differences concerning CG_DMV and CHH_DNMV due to Cu(II) concentration. Our data suggest that the differences reflect the switch from gametophytic to sporophytic pathway in 'A' missing in 'SE' and some not apparent aspects of epigenetic mechanisms affecting 'A' and 'SE'.

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Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Consent to participate Not applicable.

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Ethical approval This article does not contain any studies with human participants or animals performed by the author.

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