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Original Article

Effect of Different DNA Demethylating Agents on *In vitro* Cultures of Peach Rootstock GF 677

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Abstract

The appearance of somaclonal variability induced by *in vitro* cultivation is relatively frequent and, in some cases, provides a valuable source of new phenotypes suitable for crop improvement. Numerous studies have confirmed that these changes can be explained by alterations of DNA methylation. Interestingly, a group of chemical compounds termed 'demethylating agents' (DMT agents) enable artificial changes to be made in the DNA methylation state. Thus, these agents are theoretically able to induce new phenotypes or more favourable properties. The objective of the present study was to verify suitable conditions for the application of different DMT agents within *in vitro* protocols for micropropagation using the stone fruit rootstock GF 677 as an example. The impact of these agents on the properties of plant regenerants was evaluated, and their DNA methylation state was controlled by using an AFLP protocol based on a restriction endonuclease that differed in its sensitivity to methylated cytosines. Moreover, the effect of newly synthesised derivates was compared with that of conventional compounds with a well-documented DNA-demethylating impact. Based on the results, the suitable concentration for treatment by a DMT agent was established as approximately 50 μM. Promising results were generated using a combination of DMT agents with different mechanisms of action, such as azacytidine and dihydroxypropyladenine; under these conditions, probable synergy between methyltransferase interception by the cytosine analogue and interruption of methyl group donation by dihydroxypropyladenine significantly changed the DNA methylation state of treated plants. Regarding newly synthesised compounds, the 5,6-dihydro-5-azacytosine nucleoside showed the most promising results, which can likely be explained by its higher stability in the media used for *in vitro* cultivation.

Keywords: 5,6-dihydro-5-azacytosine; dihydroxypropyladenine; epigenetic changes; micropropagation; somaclonal variability

Introduction

Plant tissue culture can induce a number of deviations from the parental phenotype of grown plants, a phenomenon known as somaclonal variation (Larkin and Scowcroft, 1981). The occurrence of somaclonal variability is relatively frequent and, in some cases, provides a valuable source of genetic variation for crop improvement. For example, newly selected variants may exhibit resistance to biotic or abiotic factors that can lead to improved quality or higher yields (Karp, 1995; Mehta and Angra, 2000; Predieri, 2001; Unai *et al*., 2004); other valuable properties summarized by Smulders and de Klerk (2001) can also appear in regenerants. These changes can be caused by genetic mutation, but variation in the epigenome including DNA methylation changes is frequently described as a reason for this variability (Popescu *et al*., 2002; Baránek *et al*., 2010; Sáez-Laguna, 2014; Baránek *et al*., 2015). A group of chemical compounds termed 'demethylating agents' (DMT agents) enable artificial hypomethylation of DNA in treated cells. This group includes some compounds with well-documented demethylating effects, such as 5-azacytidine (AC), 5,6-dihydro-5-azacytidine (DHAC), zebularine, and (S)-9-(2,3-dihydroxypropyl)adenine (DHPA), as well as the recently identified demethylating agent alpha anomer of 5,6-dihydro-2'-deoxy-5-azacytidine (ALPHA DHDAC) (Matoušová *et al*., 2011; Krečmerová and Otmar, 2012). The application of these compounds can theoretically induce new phenotypes or more favourable properties in treated plants.

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It can also plausibly be assumed that the level of DNA methylation plays an important role in establishing some properties that are theoretically beneficial for enhancing the effectiveness of commercial *in vitro* propagation. Among some of the most important examples, reduced vitality of long-term re-used *in vitro* cultures is often observed. This decrease in vitality can be linked to increasing DNA methylation in these old cultures, while rejuvenated variants usually show decreased levels of DNA methylation (Valledor *et al*., 2007). Another advantageous effect is that rejuvenated plants exhibit greater root tissue differentiation (Smulders and de Klerk, 2011), which should theoretically improve the economics of *in vitro* regenerant rooting. Based on those premises, the application of DMT agents to *in vitro*-grown material can theoretically improve both of these factors associated with the effectiveness of *in vitro* propagation.

The most commonly used DMT compounds are cytidine analogues such as 5-azacytidine and zebularine. Once incorporated, the analogues covalently trap DNA methyltransferases and mediate their degradation, leading to passive loss of DNA methylation in the treated cells (Yoo *et al*., 2004; Stresemann and Lyko, 2008). Another compound with the power to influence DNA methylation levels is 9-(2,3-dihydroxypropyl) adenine (DHPA), which effectively interferes with the degradation of the methyl-group donor competitor, S-adenosyl-L-homocysteine (SAH). DHPA blocks the hydrolysis of SAH and therefore prevents methyltransferases from carrying out further methylation (Schanche *et al*., 1984, Kovařík *et al*., 1994).

Long-term *in vitro* cultures of the GF 677 variety were used as study material in this work. The GF 677 variety is a hybrid of *Prunus amygdalus* × *P. persica* and is commonly used as a rootstock for peach trees. There is a well-established protocol for the propagation of this particular rootstock by tissue culture, and there have been many studies involving the micropropagation of GF 677 (Dimasi-Theriou and Economou, 1995; Tsipouridis *et al*., 2005; Aghaye and Yadollahi, 2012).

The objectives of the present study were i) to determine a suitable range of concentrations for the tested group of DMT agents, ii) to evaluate the initial effect of the tested DMTs on the properties of micropropagated GF 677 rootstock plants from a morphological point of view and iii) to compare the ability of proven DNA-demethylating compounds and newly synthesised ones to change the DNA methylation state of *in vitro* cultures. To obtain the necessary data, AFLP fingerprints obtained by using the *MspI* endonuclease, which is sensitive to DNA methylation at recognized CCGG sequences, was used as a tool.

Materials and Methods

Applied DMT agents

Detailed information on the compounds used for treatment is listed in Table 1. Four DMT agents and one combination of agents that function on the basis of different demethylating principles (AC and DHPA) were added in the form of an aqueous solution to the initial growth media (details of the medium composition are provided in Chapter 2.2). Each compound was tested by using three different concentrations in the media: 10 μM, 50 μM and 100 μM. For each DMT compound and its concentration, three replicates were performed in three glass containers.

In vitro cultivation

For the cultivation of GF 677 rootstock tissue cultures, glass containers with Quoirin and Lepoivre (1977) medium (pH 6.3) enriched with the different DMT compounds were prepared. Axillar shoot explants that were uniform in both their growth and physiological properties were transferred to the media, with each glass container holding five shoots, resulting in 15 individuals per treatment. These plants were maintained less than 16/8 hour of light/dark conditions in a 21 °C growth chamber. The light intensity was 20.25 $\mu mol.m^{-2}.s^{-1}$. After 10 days in this initial media, the plants were transferred to cytokinin-enriched (1 mg IBA) Quoirin and Lepoivre multiplication media (free of DMT compounds), subsequently maintained under these conditions for 20 days and subjected to morphological trait evaluation.

Morphological evaluation

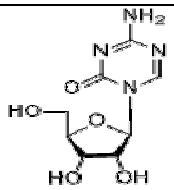
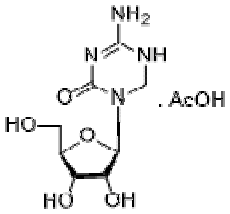
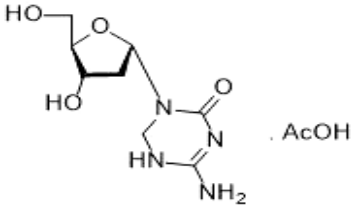
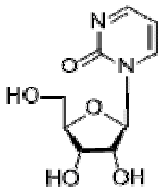
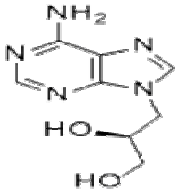
The first assessment of the effect of the drugs on the cultures was performed after 10 days in the DMT compound-enriched Quoirin and Lepoivre media. Further morphological trait evaluations were performed after 7 and 20 days in the cytokinin-enriched Quoirin & Lepoivre multiplication media (free of DMT agents). Each evaluation included photo-documentation, estimation of biomass by measuring shoot cluster size and examination of evident changes in behaviour or phenotype compared to the control (untreated) culture.

AFLP fingerprints obtained by using MspI as an endonuclease sensitive to DNA methylation

As a source for the isolation of DNA corresponding to each treatment variant, tissues were obtained from whole vitro plants, and three replicates were performed for each variant. The material was collected after 10 days in the basic Quoirin and Lepoivre media including the different DMT agents with set concentrations, resulting in a total of 18 samples including the untreated control variant. For the DHPA variants, only the samples treated with concentrations of 100 μM and 50 μM were included in the AFLP analysis due to slight bacterial infection of the 10 μM variant, which did not pose problems concerning morphological trait evaluation but could ultimately affect the MSAP results. For DNA isolation, the DNeasy Plant Mini Kit (Qiagen) was used in accordance with the manufacturer's instructions.

A 300 ng sample of isolated DNA was digested with the *MspI/EcoRI* enzyme combination. *MspI* can cleave non-methylated CCGG sequences and hemi- (^{m}C only on one DNA strand) or fully methylated C^{m}CGG sequences but not hemi- and fully methylated ^{m}CCGG and ^{m}C^{m}CGG sequences (Fulneček and Kovařík, 2014); therefore, comparison of the obtained amplicon spectra among the analysed samples allows evaluation of the degree of the changes in their DNA methylation landscape.

Table 1. List of the compounds used for the treatment

Code of the compound	Structural formula	Molar mass (M)	Supplier
5-Azacytidine (AC)		244. 21	Institute of Organic Chemistry and Biochemistry
5,6-Dihydro-5-azacytidine, acetate salt (DHAC)		306.27	Institute of Organic Chemistry and Biochemistry
Alpha anomer of 5,6-dihydro-2'-deoxy-5-azacytidine, acetate salt (ALPHA DHDAC)		290.28	Institute of Organic Chemistry and Biochemistry
Zebularine		228.20	Sigma-Aldrich
(S)-9-(2,3-dihydroxypropyl)adenine (DHPA)		209.09	Institute of Organic Chemistry and Biochemistry

The restricted fragments were ligated with *EcoRI/Msp1* adaptors. The conditions and adaptor sequences used as well as the following steps were carried out as in Baránek *et al.* (2010). The total number of primer combinations used was 6.

The distribution of amplicons within individual treatments was classified as present (1) or absent (0). The data were entered into a computer file as a binary matrix. The obtained binary matrix representing the presence or absence of individual amplicons was analysed using the Nei and Li/Dice algorithm (Nei and Li, 1979). Based on the coefficients of similarity calculated by the UPGMA method, a dendrogram was created using FreeTree software (Hampl *et al*., 2001).

Results

Morphological evaluation

The results of the assessment (Table 2) showed a retardation effect of some of the tested compounds, where 5-azacytidine (AC) exerted the strongest influence. Surprisingly, the combined AC+DHPA treatment group was not hindered by significant growth inhibition, and the growth rate of the treated plants was comparable with that of the control and the DHPA-treated plants in the first term of evaluation. All observed deviations from the control occurred in a concentration-dependent manner, with higher concentrations making the variations more pronounced. In most cases, after the initial growth decline, the plants grown in the 10 μM media exhibited similar growth intensity to the control plants 20 days after transfer to multiplication media. In the case of the 100 μM concentration, the growth inhibition effect seemed to persist, and the plants showed a continuous decrease in biomass production throughout the entire morphological trait assessment period (i.e., for 30 days after the application of DMT agents). No notable changes in phenotype were detected during the evaluation period, except for larger or slightly curled leaves in the DHPA-treated variant, as indicated in Table 2.

Comparison of AFLP fingerprints obtained by using Msp1 as an endonuclease sensitive to DNA methylation

The total number of evaluated amplicons was 594. All treated plants showed significant differences in their DNA

Table 2. Results of morphological evaluation

Used compound	Concentration	Biomass evaluation (% of control) – variant A*	Biomass evaluation (% of control) – variant B**	Biomass evaluation (% of control) – variant C***
		Other effect	Other effects	Other effects
AC	10 µM	60	70	70
	50 µM	50	60	50
	100 µM	50	50	40
DHAC	10 µM	90	100	100
	50 µM	80	80	80
	100 µM	70	70	60
ALPHA DHDAC	10 µM	80	90	100
	50 µM	80	80	70
	100 µM	70	60	50
DHPA	10 µM	90	90 Bigger leaves	70 Less shoots, bigger leaves
	50 µM	80	90 Bigger leaves	80 Less shoots, bigger leaves
	100 µM	100 Bigger leaves	100 Curled leaves	90 Curled leaves
Zebularine	10 µM	80	80	90
	50 µM	60	60	60
	100 µM	50	50	40
AC+DHPA	10 µM	90	100	100
	50 µM	90	80	70 Less shoots
	100 µM	90	80	60 Smaller growth
Control	-	100	100	100

* biomass of the plants after 10 days on media with respective DNA demethylating compound
** biomass of the plants 7 days after the transfer from media with respective demethylating compound on multiplication media
*** biomass of the plants 20 days after the transfer from media with respective demethylating compound on multiplication media

methylation landscape in comparison with the control, whereas the average coefficient of MSAP spectrum similarity between all variants was computed as 0.6124. Regarding the strength of the ability of individual compounds to change the DNA methylation landscape, the lowest coefficients of similarity on average were obtained for variants derived from DHAC treatment compared with the untreated control. In fact, the average similarity under the 10, 50 and 100 µM DHAC concentrations was 0.5654.

The dendrogram constructed on the basis of mutual similarity coefficients (Fig. 1) showed that the variants could be categorized into three distinct groups. The group showing highest similarity with the control variants was mainly composed of AC-treated variants from all three tested concentrations and two variants obtained under zebularine treatment.

Another branch of the dendrogram comprised the ALPHA DHDAC-treated samples from the whole concentration spectrum and the 100 µM DHPA-treated samples, indicating the pronounced effect of the DMT agent concentration. The last group, located in the upper part of the dendrogram, included variants showing the greatest distinctness of their methylation status compared to the control. It was not surprising that variants obtained under the DMT agent combination of AC+DHPA were also included in this cluster, confirming their theoretical synergic effect on the hypomethylated genome of the treated tissues. Interestingly, the samples treated by all concentrations of DHAC were also included in this cluster, indicating that this compound is highly promising for *in vitro* applications in experiments focused on decreasing DNA methylation status in treated tissue.

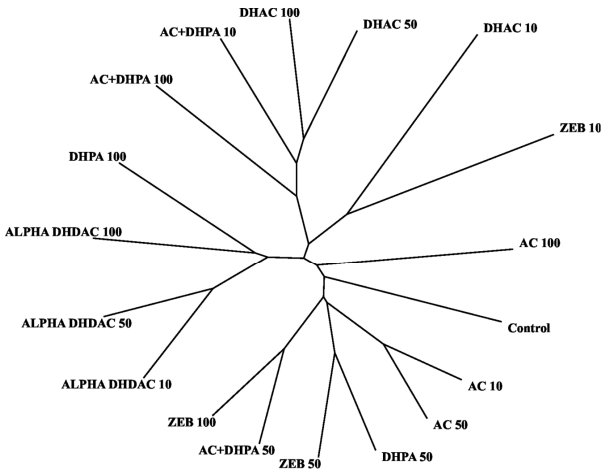


Fig. 1. Dendrogram showing the similarity of AFLP spectra generated by using the methylation-sensitive *Msp*I endonuclease

Discussion

The main purposes of this work were to verify conditions suitable for the application of DMT agents and their effect on *in vitro* cultures as potential inducers of beneficial changes and to compare some traditional and newly designed DNA-demethylating compounds. The results of the morphological analysis showed a significant number of changes compared to the untreated control. The most frequent of these changes was growth retardation, which was most prominent when using AC. However, the results of AFLP analysis of the modulation of the DNA methylation landscape grouped the plants treated with AC with the control variant (Fig. 1), indicating that the differences in growth rates may not have been due to the demethylating effect of the drug. This finding was not surprising in light of the information that in addition to the ability to be incorporated into DNA and inhibit its methylation, AC is also incorporated into RNA and inhibits protein synthesis (Fučík *et al*., 1970; Diesch *et al*., 2016) while decreasing the stability of polyribosomes and the maturation of rRNA (Reuveni and Rosenthal, 1979). Furthermore, AC has been indicated to be quite unstable in aqueous solutions and is sensitive to light and oxidation (Notari and DeYoung, 1975; Diesch *et al*., 2016). The delay of growth and other differences observed in comparison with the control may therefore have been caused by other mechanisms of change that are independent of DNA methylation status and are therefore not measurable by MSAP analysis.

On the other hand, although zebularine exhibits a much longer half-life under physiological conditions and fewer side effects than AC (Cheng *et al*., 2003), zebularine can induce DNA damage during strand synthesis in DNA replication, triggering a DNA damage response independent of changes in DNA methylation status (Liu *et al*., 2015). The grouping of variants treated by zebularine within the generated dendrogram (Figure 1) indicated that zebularine had a relatively small effect on DNA methylation. However, zebularine was previously proven to induce a significant decrease in cytosine methylation (Baubec *et al*., 2009), which is seemingly in direct opposition to the results obtained in the present study. One possible explanation for this disparity could be the different experimental backgrounds between the compared experiments. Within the present study, compounds with stronger demethylating effects were used, making zebularine substandard within this experiment, although it was still strong enough to be described as very effective, as indicated by Baubec *et al*. (2009).

In the case of the sub-cluster including the variants that deviated most from the control (upper part of Fig. 1), the combined effect of AC+DHPA seemed to introduce the most prominent changes in the DNA methylation patterns of the treated plants, together with DHAC. The enhanced effect of DHAC can be explained by the better physical properties of this compound, such as its better solubility in aqueous solutions or better stability in agar media. In fact, DHAC and ALPHA DHDAC differ from the classic 5-azacytidine nucleosides in the saturation of the double bond at position 5,6 in their structure. This difference results in significant improvement of their stability as well as an increase in their basicity. Therefore, these compounds are prepared as salts with acids (as a salt of acetic acid in this case). Interestingly, the second substance with the 5,6-dihydro modification, ALPHA DHDAC, also showed promising results in terms of its demethylating effect. As can be seen from the dendrogram, treatment with both 5,6-dihydro-5-azacytosine compounds exerted a stronger effect than treatment with commonly used DMT agents, such as zebularine or AC. Based on this finding, 5,6-dihydro-5-azacytosine compounds can be recommended for use in experiments focused on decreasing the DNA methylation state in plant tissues maintained under *in vitro* conditions. However, the realization of this strategy can be difficult due to the limited availability of this compound. Another possibility for inducing a relatively strong DNA-demethylating effect is to use the combination of AC+DHPA, as shown by the presented results.

The obtained results may also be generally important in other scientific arenas where the use of effective DNA-demethylating agents is highly desirable, such as environmental studies focused on the transgenerational effects of plant adaptation to stresses, in which DNA methylation probably plays a role (Latzel *et al*. 2016; Gonzáles *et al*., 2017), and studies focused on the phenomenon of plant inbreeding depression (Vergeer and Wagamaker, 2012), gene expression (Bossdorf *et al*., 2010; Kiselev *et al*., 2011) or the rate of point mutations (Kiselev, 2019).

Conclusions

The obtained results indicate the importance of the concentration of DMT compounds, as the morphological and growth rate differences increases with the dosage if compared with the untreated control. Based on the presented results, the optimal concentration for DMT agent treatment is approximately 50 μM, where the effect on methylation levels is clear, but no significant retardation of growth is observed. Promising results from the perspective of the DNA-demethylating effect were generated using a combination of DMT agents with different mechanisms of action, such as azacytidine and dihydroxypropyladenine (DHPA), where probable synergy between methyltransferase interception by the cytosine analogue and interruption of methyl group donation by DHPA changed the DNA methylation state of the treated plants. Regarding newly synthesised compounds, the 5,6-dihydro-5-azacytosine (DHAC) nucleoside showed the most promising results, which can likely be explained by its higher stability in the media used for *in vitro* cultivation.

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Conflicts of interest

The authors declare that there are no conflicts of interest related to this article.

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