Inhibitors of DNA methylation and histone deacetylation impact the regenerative capacity of parsnip protoplasts

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Introduction

Epigenetic silencing is a natural phenomenon in which the expression of genes is regulated through modifications of DNA, RNA, or histone proteins. DNA methylation and histone deacetylation are considered major determinants of epigenetic silencing, as they lead to chromatin modifications resulting in a decreased transcriptional availability of genes. Protoplasts serve as a unique tool for a range of procedures focused on overcoming naturally occurring sexual incompatibility barriers and an efficient genetic transformation and genome editing of plant cells.

Parsnip is a monocarpic biennial crop of growing economic significance. Therefore, it is important to develop an efficient and reproducible method for regenerating plants from protoplasts of this species.

The aim of the study

To evaluate the effect of inhibitors of:

- DNA methylation
- histone deacetylation

on the regenerative ability of protoplasts derived from two parsnip cultivars.

For this purpose, mesophyll-derived protoplasts were treated with either azacytidine (azaC) or vorinostat (SAHA).

Results

- Highly chloroplast-rich protoplasts (**fig. 1A**) were successfully isolated from the leaves of 3-week-old plant cultures in both cultivars, reaching on average 4.02 ± 0.4 x 10^6 protoplasts per g FW (**fig. 2A**). No significant differences in the protoplast yield were observed between the analysed cultivars.

- Protoplasts stained with FDA (**fig. 1B-C**) five days after embedding in alginate matrix exhibited a moderate level of viability, yielding on average 56.9 ± 3.4% (**fig. 2B**). Protoplast cultured in PPC6 and KM medium were more viable (78.7 and 75.4%, respectively), than in KM with addition of azaC or SAHA (35.6-39.7% and 51.5-60.2%, respectively).

- The first cell divisions occurred between the 7th and 10th day of the protoplast culture. The formation of multi-cell aggregates was observed in all culture variants (**fig. 1D**). The mean plating efficiency reached 13.3 ± 1.1% in the 20th day of the culture, and was influenced by both the accession (**fig. 2C**) and the composition of the culture medium (**fig. 2D**). Compared to PPC6 medium, KM slightly increased plating efficiency (17.1% to 19.5%). The addition of azaC and SAHA has dramatically decreased plating efficiency (**fig. 2D**) for both cultivars.

- Both cultivars showed the ability to form proembryogenic mass, followed by the development of somatic embryos on the BI + PSK solid medium (**fig. 1E, F**). In total, 1170 somatic embryos have developed from calli cultures obtained from four independent protoplast isolations within one to five months **(tab. 1)**. Majority of embryos were characterised by abnormal development (e.g. absence of apical/root meristems, single/multiple cotyledons, loss of bipolarity and hyperhydricity; **fig. 1G**). Only three embryos regenerated into morphologically normal plants and two were successfully acclimatised into the greenhouse conditions (**fig. 1H**).

Table 1. Regeneration and acclimatization efficiency of parsnip plants regenerated from callus, cultured for one to five months

Cultivar	Culture variant	Age of callus culture	Developed embryos	Developed plantlets	Regenerated plants	Acclimatized plants
'Półdługi biały'	KM + PSK	1	603	25	0	0
'Półdługi biały'	azaC 25 µM	2	215	8	1	0
'Sabre F1'	KM + PSK	3	165	19	2	2
'Sabre F1'	SAHA 0.075 µM	5	187	32	0	0

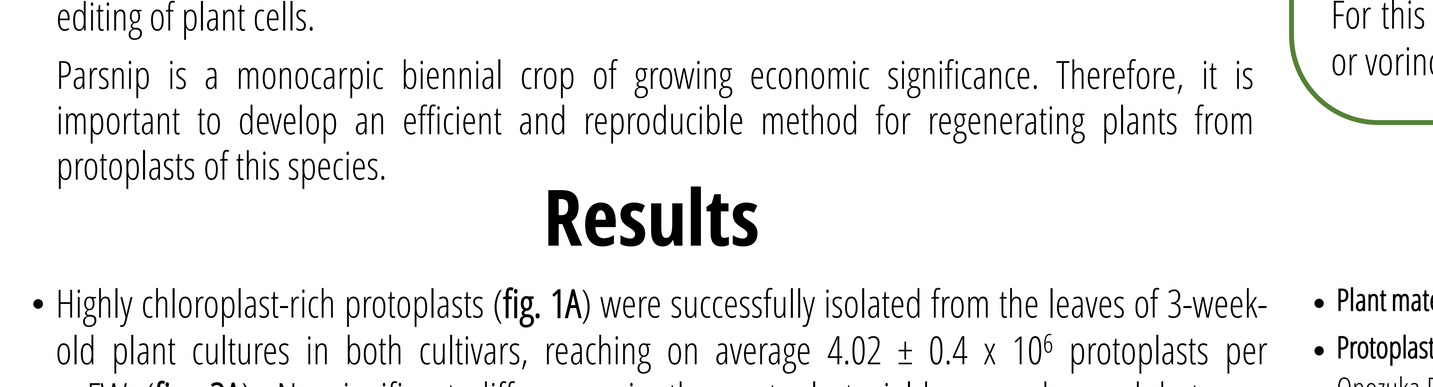

Figure 1. Plant regeneration from leaf-derived protoplasts of parsnip. **A** – leaf protoplasts, **B, C** – green fluorescence (C) of viable protoplasts (B) after FDA staining, **D** – a multi-cell protoplast-derived aggregate in 20th day old culture, **E** – proembryogenic mass (arrow), **F** – somatic embryos, **G** – abnormally developing embryos (arrows) , **H** – completely regenerated plant of 'Sabre'. Scale: **A-D** 50 µm; **E-G** 1 mm.

Material and methods

- **Plant material**: 2 cultivars of parsnip ('Półdługi biały' and 'Sabre' F1)
- **Protoplast isolation and culture**: 3-week old in vitro grown seed-derived plants; 16 h enzymatic cell wall lysis (cellulase Onozuka R-10 and pectolyase Y-23); protoplast purification by gradient centrifugation; immobilization of cells in alginate matrix; protoplast culture in the PPC6 medium (Stelmach and Grzebelus 2023) with the addition of phytosulfokine (PSK; 100 nM), modified (increased osmotic pressure) KM medium (Kao and Michayluk 1975) with PSK, and KM + PSK + azaC (25 µM or 75 µM) or KM + PSK + SAHA (0.025 µM or 0.075 µM);
- **Plant regeneration**: performed in the 60th day of the protoplast culture; alginate depolymerization followed by washing in CPPD medium (Dirks et al. 1996); calli culture on BI medium supplemented with PSK (100 nM); transfer of developed somatic embryos onto solid R medium;
- **Plant acclimatisation**: transfer of the regenerated plants into peat substrate in controlled conditions (20°C, 90% humidity for 2 weeks then 70% humidity for 2 weeks); 90-day old plants were moved into the greenhouse conditions;
- **Data collection**: protoplast yield presented as the number of protoplasts per gram fresh weigh (FW) of source tissue; viability of protoplasts by staining with fluorescein diacetate (FDA) in the 5th day of culture; plating efficiency on the 20th day of culture – the ability of single cells to perform mitotic divisions and to create cell aggregates; three to six independent protoplast isolation experiments were carried out; the overall effect of treatments was determined using multivariate analysis of variance (ANOVA) and the Tukey-Kramer HSD test was used for the separation of means.

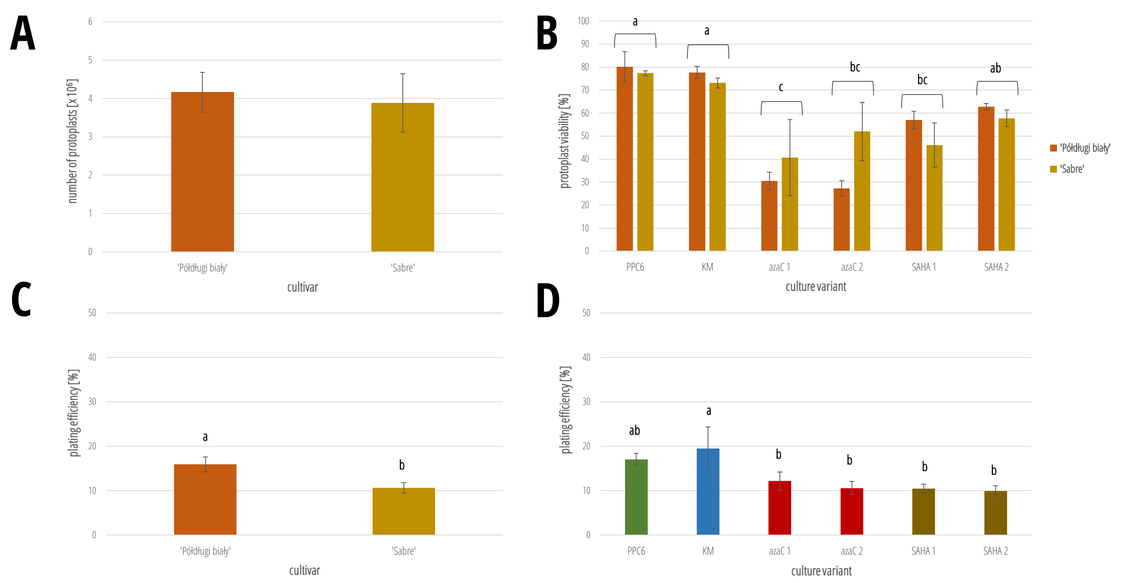

Figure 2. **A** – the mean protoplast yield obtained from two parsnip cultivars; **B** – viability of leaf-derived protoplasts of parsnip cultivars in the 5th day after isolation; **C** – the mean plating efficiency in the 20th day of culture, **D** – the mean plating efficiency in the 20th day of culture depending on the culture medium variant. *PPC6* – parsnip protoplast culture medium with 100 nM of PSK; *KM* – mod. Kao & Michayluk medium with 100 nM of PSK; *azaC 1, azaC 2, SAHA 1, SAHA 2* – KM + PSK with azaC 25 µM, azaC 75 µM, SAHA 0.025 µM or SAHA 0.075 µM, respectively. Bars show standard error. Means with different letters are significantly different at 0.05 probability

Conclusions

Modified KM medium with added PSK facilitated somatic embryogenesis in both parsnip cultivars.

Azacytidine and SAHA significantly decreased the viability of protoplasts and the plating efficiency of cultures. Additionally, they did not enhance the formation of somatic embryos compared to KM medium with PSK.

The majority of developed somatic embryos were abnormal and did not regenerate into plants.

References

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This research was supported by the Polish Ministry of Agriculture and Rural Development