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ORIGINAL RESEARCH PAPER

Early studies on the effect of peptide growth factor phytosulfokine-α on *Brassica oleracea* var. *capitata* L. protoplasts

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Abstract
Phytosulfokines (PSK) are peptidyl growth factors with the potential of inducing cell proliferation. We examined the effect of supplementation of liquid culture medium with 0.1 μM phytosulfokine-α (PSK-α) on protoplast viability and division frequencies in seven accessions of *Brassica oleracea* var. *capitata* L., including cultivars and breeding lines. Protoplasts were isolated from leaves and hypocotyls of in vitro grown plants and immobilized in calcium-alginate layers. Cabbage protoplast-derived cells cultured in medium supplemented with 0.1 μM of PSK-α had higher viability and division frequencies compared to cells cultured in PSK-α-free control medium. The effect of PSK-α was more pronounced in low-responding accessions ('Sława z Gołębiewa', 'Ramkila F1', LM, and LM98); however, in two cultivars with very low response ('Badger Shipper' and 'Oregon 123'), although the division frequencies in the media supplemented with PSK-α were increased over the control, the differences were not significant. Obtained callus colonies were subjected to regeneration. PSK-α supplemented into the liquid culture medium had an indirect effect on shoot regeneration by inducing sustained cell divisions leading to an increase in shoot regeneration in Sława z Gołębiewa and both breeding lines.

Keywords
cabbage; peptide; phytosulfokine; PSK; protoplast culture

Introduction

Brassica oleracea var. *capitata* L. belongs to the family Brassicaceae, which includes many economically important vegetables such as broccoli, cauliflower, or brussels sprouts. Protoplast cultures have been intensively studied in the genus *Brassica*, with special emphasis on *Brassica napus* [1–3]; however, in *B. oleracea* var. *capitata*, positive results have been achieved using selected accessions with high division frequencies and regeneration capacity [4–6]. The effect of genotype has overridden many efforts to use *B. oleracea* var. *capitata* protoplast cultures in biotechnology [5,7,8]; therefore, it is crucial to develop improvements in protocols leading to an increase in mitotic activity and regeneration process.

Plant hormones, such as auxins and cytokinins, have been considered to be the main growth regulators involved in cell divisions [9]. In several studies, *B. oleracea* protoplasts were cultured in liquid media containing various proportions of auxins and cytokinins; however, in some genotypes, despite the presence of plant growth regulators, divisions of protoplast-derived cells were very low or the cells failed to divide [6,10–12]. Mitotic activity of protoplast-derived cells can also be improved by the application of a nurse culture, feeder layers, or conditioned media with addition of filtrate prepared from a suspension culture of rapidly dividing cells [13]. Chen and others [14] described a procedure for regenerating shoots from hypocotyl-derived protoplasts of *B. oleracea* var *capitata* f. *rubra* in the presence of viable protoplasts of *B. juncea*. Another example

is the use of feeder layers from *B. campestris* suspension cultures for protoplast culture of *B. oleracea* ssp. *botrytis* and *B. oleracea* ssp. *italica* [15]. Such an approach might be beneficial; however, it is laborious and time consuming and requires the establishment of actively dividing suspension cultures [16]. Favorable effect of nurse cultures, feeder layers, or conditioned media suggested the presence of division-inducing factors that are secreted into the medium with cultured cells [17,18]. Many efforts have been made to identify and characterize such factors and initial amino acids, purine derivatives, highly hydrophilic and heat stable agents, or protease-resistant small compounds were considered as putative mitogenic inducers; however, the available assay methods were not sufficiently sensitive to define any specific compound [19–21]. The elaboration of highly sensitive bioassay systems for the characterization of mitotic activity in low-density cultures of asparagus in the presence of conditioned medium led to the discovery of phytosulfokines (PSK), biologically active components with mitogenic activity [22]. Phytosulfokines PSK-α and PSK-β are small (five and four amino acids, respectively) peptides containing two post-translationally sulfated tyrosine residues. Matsubayashi and others [23] reported that PSK-β is an enzymatically degraded product of PSK-α. They also observed that the unsulfated PSK-α analogue was less active than the native form of PSK-α. Moreover, the studies of Bahyrycz and others [24] showed that PSK analogues with different modifications in the position of the sulfur atoms had weak or no biological effects. These results point out that sulfur plays an important role in the creation of biological properties of phytosulfokine. PSK-α is a primary signal molecule responsible for cell to cell communication [25]. Studies in *Arabidopsis thaliana* on PSK signaling revealed two genes (*PSKR1* and *PSKR2*) encoding for PSK receptors. Analysis of *pskr1-3* and *pskr2-1* T-DNA insertion lines showed that root elongation was predominately controlled through *PSKR1*. *PSKR1* signaling regulated root growth mainly by increasing cell size [26]. Studies on *A. thaliana* hypocotyl-derived protoplasts showed that PSK signaling controls osmotically-driven cell expansion suggesting that PSK-α acts as an osmoregulator and involves K^+ uptake [27].

It has been reported that PSK-α plays an important role in plant growth and development due to its contribution to the formation of somatic embryos [28,29], its role in adventitious buds and roots formation [25,30], and its contribution to cellular longevity and the cell division cycle [31,32]. The objective of this study was to analyze the effect of PSK-α on viability and division frequencies of *Brassica oleracea* protoplast-derived cells embedded in calcium alginate layers.

Material and methods

Plant material

As protoplasts source, we used seven accessions of *B. oleracea* var. *capitata*, including five cultivars: 'Sława z Gołębiewa' (PlantiCo, Poland), 'Reball F1' and 'Ramkila F1' (Syngenta Seeds, Poland), 'Badger Shipper' and 'Oregon 123' (Warwick Genetic Resources Unit, United Kingdom), and two breeding lines: LM and LM98 (KHiNO Polan, Poland). Seeds of all accessions were sterilized in 70% (v/v) ethanol for 2 min, 10% (w/v) chloramine T (Biochemie Gmbh, Poland) for 20 min, and washed three times with sterile distilled water for 5 min each. Seeds were germinated on MS [33] medium supplemented with 0.8% (w/v) agar (Biocorp, Poland). Prior to isolation of protoplasts from leaves, seeds were placed in sterile 500-mL plastic boxes (Pakler Lerka, Poland) containing 80 mL of MS medium and kept at 26 ±2°C under a 16-h photoperiod with light intensity 55 μmol m^{-2} s^{-1}. Etiolated hypocotyls were obtained from seeds placed in a 90-mm Petri dish with 25 mL of MS medium and kept in the dark at 26 ±2°C.

Protoplast isolation and culture

Young leaves of 4-week-old plants and hypocotyls from 2-week-old seedlings were used as donor tissue for protoplast isolation. Protoplasts were isolated and cultured according to the protocol described by Kiełkowska and Adamus [10]. The enzyme solution for

tissue digestion consisted of 0.5% (w/v) cellulase Onozuka R-10 (Duchefa Biochemie, the Netherlands), 0.1% (w/v) pectolyase Y-23 (Duchefa Biochemie), 3 mM $CaCl_2$, 2 mM of 2-(*N*-morpholino) ethanesulfonic acid (MES; Sigma-Aldrich, Poland), and 0.4 M mannitol, and was filter-sterilized (0.22 µm; Millipore, United Kingdom). Isolated and purified protoplasts were immobilized in filter-sterilized alginate according to the protocol of Kiełkowska and Adamus [34]. Final density of protoplasts was 4×10^5 per mL of culture medium. Alginate discs with embedded protoplasts were transferred to 60-mm Petri dishes containing 4 mL of culture medium. Basal culture medium was CPP medium according to Dirks et al. [35], supplemented with 0.1 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg L^{-1} zeatin, pH 5.6, filter sterilized (0.22 µm; Millipore). Basal medium was supplemented with 0.1 µM of PSK-α (Peptide Institute, Inc., Japan). Alginate discs with embedded protoplasts cultured in basal medium were used as a control. Culture medium was renewed after 10 days and cultures were incubated in the dark at 26 ±2°C.

Protoplast viability was estimated on the first and fifth day after isolation by staining with fluorescein diacetate (FDA) according to Anthony and others [36]. Observations of yellow-green fluorescence of viable cells were performed using an Axiovert S 100 (Carl Zeiss, Germany) inverted microscope.

Shoot regeneration

Protoplast-derived callus colonies were freed from alginate layers according to the protocol of Damm and Willmitzer [37] and transferred for further development into 90-mm Petri dishes with 20 mL of regeneration medium consisting of B5 [38], micro-, macroelements and vitamins with 750 mg L^{-1} $CaCl_2 \cdot 2H_2O$, 1 mg L^{-1} benzylaminopurine (BA), and 20 g L^{-1} sucrose. The medium was adjusted to a pH of 5.7–5.8 and 0.28% (w/v) Gelrite was added (Sigma-Aldrich) prior to autoclaving (20 min at 121°C; 0.1 MPa). Developing shoots were transferred to fresh medium every 3 to 4 weeks. Rooting was performed on MS [33] medium with 1 mg L^{-1} of indoleacetic acid (IAA). Cultures were maintained at 26 ±2°C with a 16-h photoperiod at a light intensity of 55 µmol m^{-2} s^{-1}.

Statistical analysis

The single experiment consisted of four independent protoplast isolations with a single treatment represented by four Petri dishes. Experiment was repeated three times. Viability of protoplasts was calculated as the number of protoplasts with yellow-green fluorescence divided by the total number of observed cells (× 100). Division frequency was expressed as the number of dividing protoplast-derived cells per total number of observed cells (× 100). Regeneration frequency was calculated as the number of shoots regenerated from callus per total number of calli cultured on the regeneration medium (× 100). Analyses were performed using Statistica ver. 10.0 (Statsoft, Inc., Poland) with the ANOVA module. Mean separation was performed with Tukey's honestly significant difference (HSD).

Results

Protoplast viability

On the day following isolation, protoplasts of majority of tested accessions ('Oregon 123', 'Reball F1', 'Badger Shipper', and breeding line LM) had relatively high (88–98%) viability, irrespectively from the presence of PSK-α in the medium (Fig. 1). In 'Sława z Gołębiewa' and 'Ramkila F1', higher viability (respectively 91% and 86%) was observed in protoplasts cultured in PSK-free control medium. The lowest protoplast viability was observed in breeding line LM98; however, protoplasts of this accession cultured in the presence of 0.1 µM of PSK-α were more viable (82%) in comparison with protoplasts

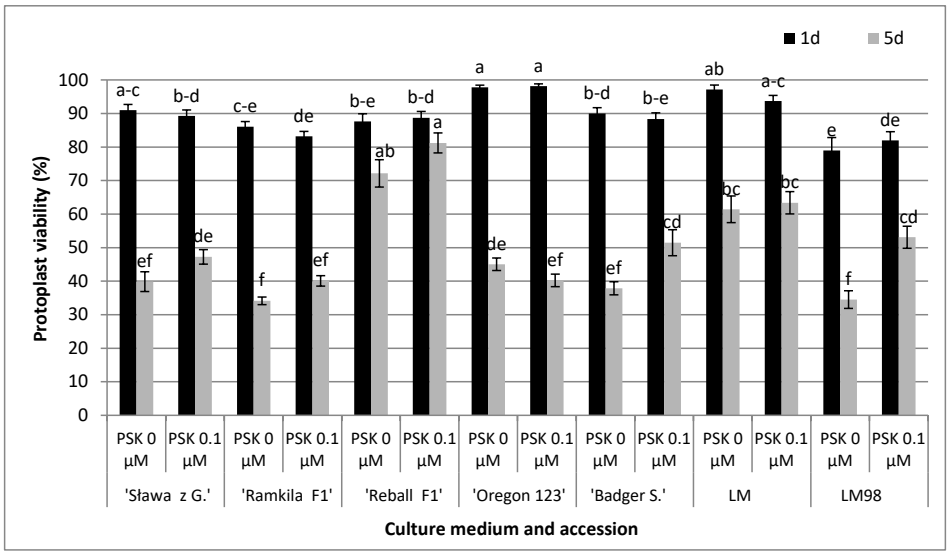

Fig. 1 Effect of phytosulfokine-α and accession on the viability of *B. oleracea* protoplast-derived cells in the first and fifth day of culture. Bars represent means for leaf- and hypocotyl-derived cells jointly ±*SE*. Black and grey bars should be considered separately. Values followed by the same letter are not significantly different ($p \leq 0.05$, HSD).

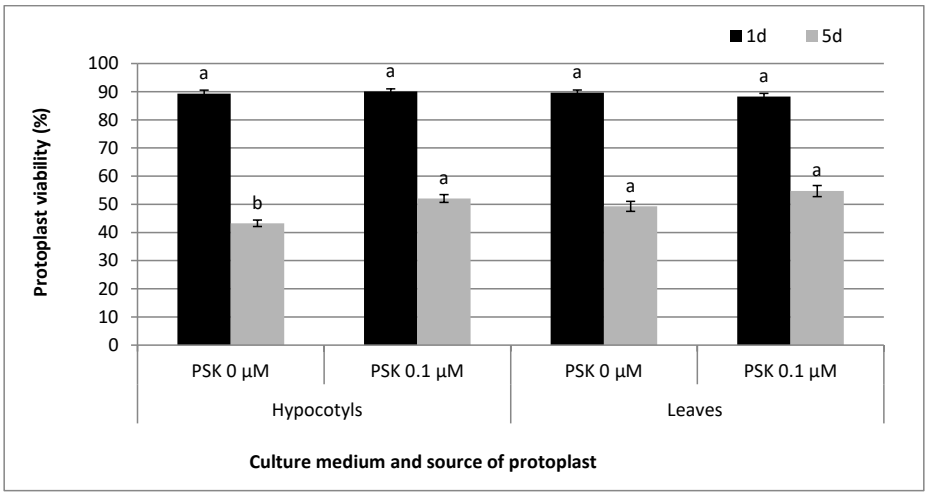

Fig. 2 Effect of phytosulfokine-α and protoplast source on the viability of *B. oleracea* protoplast-derived cells in first and fifth day of culture. Bars represent means ±*SE*. Black and grey bars should be considered separately. Values followed by the same letter are not significantly different ($p \leq 0.05$, HSD).

cultured in control medium (79%). On the fifth day of culture, the number of viable cells decreased in all tested accessions. At this period of time, the highest percent (82) of viable cells were observed in 'Reball F1' on the medium with PSK-α; moreover, in this accession, the decrease of viability in relation to the first day of culture was lowest (7%). The highest decrease was observed in 'Oregon 123' on both tested media, where cell viability on the fifth day was approximately 55% lower than on the first day of culture. In 'Sława z Gołębiewa', 'Ramkila F1', and 'Badger Shipper', the highest decrease in cell viability (approximately 51–52%) was observed in the control medium. In these accessions, as well as in breeding line LM98, protoplasts cultured in the medium supplemented with PSK-α were more viable.

On the first day of culture, we observed no differences in viability between leaf- and hypocotyl-derived protoplasts nor the protoplast cultured in the presence of PSK-α and in control medium (Fig. 2). On the fifth day of culture, leaf-derived cells had similar viability on both tested media; however, hypocotyl-derived cells cultured in the medium supplemented with PSK-α (52%) were more viable compared to PSK-free control medium (43%).

Division frequency

Mitotic divisions were observed in all tested accessions, albeit with various frequencies, depending on the PSK-α presence in the culture medium (Fig. 3). On the fifth day of culture, the average division frequencies in all accessions did not exceed 15% (Fig. 3a). The highest division frequency was observed in breeding line LM98 (15%) and 'Sława z Gołębiewa' (14%) on the medium supplemented with 0.1 μM of PSK-α, while lowest one was found in 'Ramkila F1' (0.3%) on the control medium. At this period of time, protoplast-derived cells of 'Badger Shipper' cultured on the PSK-free control medium, did not divide; however, on the medium with PSK-α 0.5% of cultured cells underwent mitosis.

On the fifteenth day of culture, the division frequencies in all tested accessions increased (Fig. 3b). Supplementation of the culture medium with PSK-α showed a beneficial effect on mitotic activity of protoplast-derived cells in the majority of the tested accessions in comparison with the control. On the fifteenth day of culture, the highest division frequency of protoplast-derived cells was observed for 'Reball F1' in the medium supplemented with PSK-α (56%), which was approximately 10% higher than that in the PSK-free control. The protoplast-derived cells of breeding line LM98 cultured in control medium divided with a frequency of 5%, while in the medium supplemented with PSK-α 47% of protoplast-derived cells underwent divisions. The stimulating effect of PSK-α was also recorded for 'Ramkila F1', 'Sława z Gołębiewa', and breeding line LM, where the mitotic activity of protoplast-derived cells cultured in the

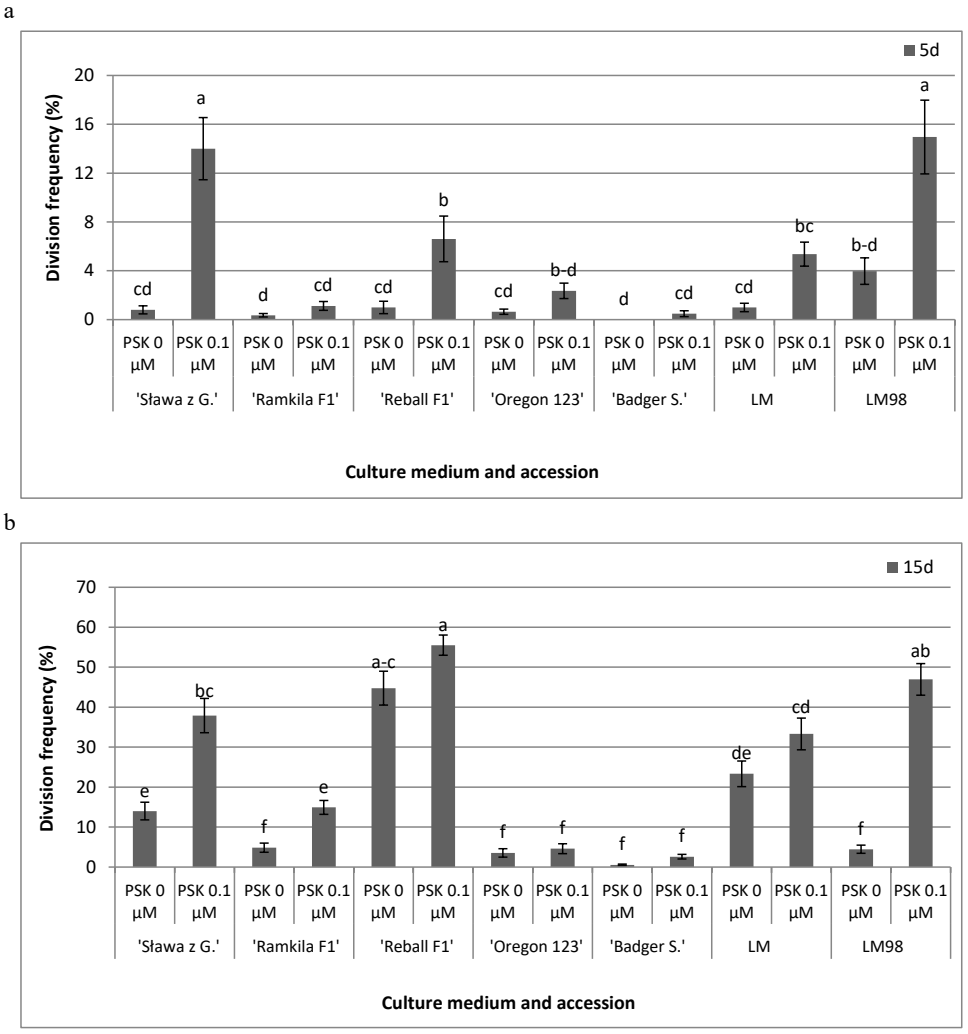

Fig. 3 Effect of phytosulfokine-α and accession on division frequency of *B. oleracea* protoplast-derived cells in the fifth (**a**) and fifteenth (**b**) day of culture. Bars represent means for leaf- and hypocotyl-derived cells jointly ±*SE* . Values followed by the same letter are not significantly different ($p \leq 0.05$, HSD).

control medium was 5%, 14%, and 23%, respectively, while activity of cells cultured in the medium supplemented with PSK-α was 15%, 38%, and 33%, respectively. For two cultivars, 'Oregon 123' and 'Badger Shipper', although the division frequencies of protoplast-derived cells cultured in the media supplemented with PSK-α were slightly increased compared to the control, the differences were not significant.

On the fifteenth day of culture, higher division frequency was observed among hypocotyl-derived cells cultured in the media supplemented with PSK-α (33%) compared to the PSK-free control (10%) (Fig. 4). Similar tendency was observed for leaf-derived cells, although frequencies of divisions were lower.

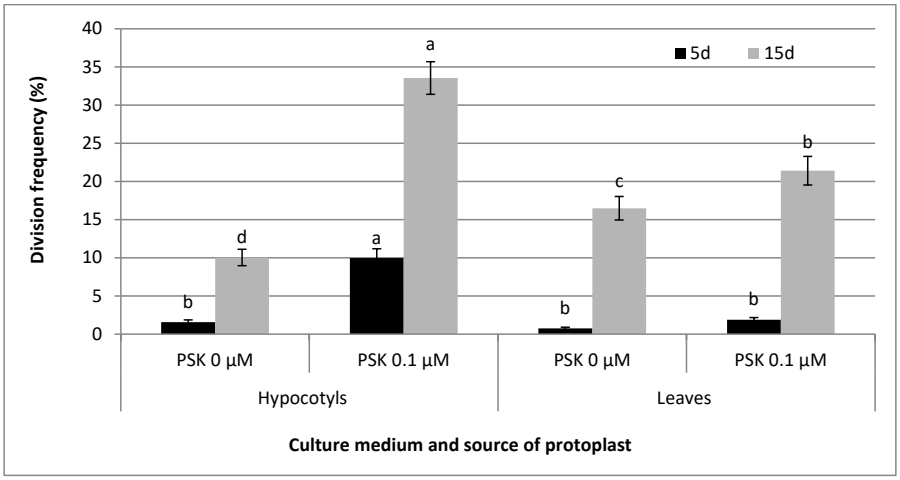

Fig. 4 Effect of phytosulfokine-α and protoplast source on the division frequency of *B. oleracea* protoplast-derived cells in the fifth and fifteenth day of culture. Bars represent means ±*SE*. Black and grey bars should be considered separately. Values followed by the same letter are not significantly different ($p \leq 0.05$, HSD).

Tab. 1 Shoot regeneration from the protoplast-derived callus colonies of *B. oleracea*.

Factor	Shoot regeneration (% ±*SE*)
Accession	
'Sława z Gołębiewa'	6.7 ±2.9 [b]
'Ramkila F1'	0.0 ±0.0 [c]
'Reball F1'	20.9 ±2.9 [a]
'Oregon 123'	0.0 ±0.0 [c]
'Badger Shipper'	0.0 ±0.0 [c]
LM	7.1 ±3.1 [b]
LM98	5.7 ±1.5 [b]
Source of Protoplasts*	
Leaves	8.3 ±1.8 [a]
Hypocotyls	12.1 ±3.1 [a]

Values of the factor Accession represent means for leaf- and hypocotyl-derived cells jointly. Values followed by the same letter are not significantly different ($p \leq 0.05$, HSD).
* Calculation based only on accessions with regeneration.

Shoot regeneration

Mitotic divisions of cultured protoplast-derived cells resulted in formation of multiple cell colonies, visible with the naked eye after approximately 4 weeks of culture. Colonies of calli were freed from alginate layers and placed on a solid regeneration medium. Although callus colonies were noted in all seven tested accessions, only in four of them the divisions were sustained, leading to shoot development (Tab. 1). The callus colonies of 'Ramkila F1', 'Oregon 123', and 'Badger Shipper' failed to regenerate. The highest regeneration (21%) was observed in 'Reball F1'. There were no differences in shoot regeneration between 'Sława z Gołębiewa' (7%) and breeding lines LM (7%) and LM98 (6%). We also observed no differences in shoot regeneration between protoplast sources.

Detailed analysis of shoot regeneration capacity in dependency from the presence or absence of PSK-α in the earlier cultivation stages (in the liquid medium for culturing the protoplasts) showed that for the high-responding 'Reball F1', the effect of PSK-α was insignificant (Fig. 5). In all remaining responsive accessions, regeneration capacity was higher among callus colonies derived from protoplasts cultured in medium supplemented with PSK-α. In 'Sława z Gołębiewa' and breeding line LM, the increased shoot regeneration was twofold higher compared to the control. In breeding line LM98, regeneration frequency of callus colonies developed from protoplasts cultured in the control media was 0.3%; however, from PSK-α supplemented cultures, regeneration frequency increased up to 11%.

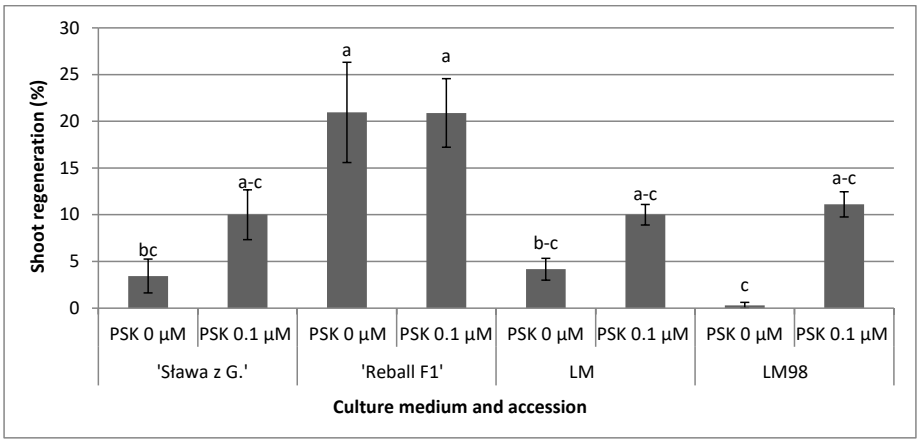

Fig. 5 Effect of phytosulfokine-α and accession on shoot regeneration from *B. oleracea* protoplasts. Bars represent means for leaf- and hypocotyl-derived cells jointly ±*SE*. Values followed by the same letter are not significantly different ($p \leq 0.05$, HSD).

Discussion

Recently, peptides have been recognized as signaling molecules playing an important role in plant cell growth and development [31,39]. Results showed that in planta, PSK signaling participates in the control of shoot and root growth. Hypocotyl elongation and root growth are promoted by PSK, mainly through the signaling of cell elongation rather than cell division [26,27]. However, in suspension cultures PSK promotes the proliferation of cells of both monocot and dicot plants [23,28,31,32] and due to this feature, phytosulfokine-α has been proposed as a candidate to supplementing plant in vitro cultures together with other well-known plant growth regulators [40,41]. These results were the assumption to perform our experiments. In our previous work, we have developed a simple and efficient protocol for *Brassica oleracea* protoplast isolation and culture with embedding protoplasts in calcium alginate layers [10,34]; however, using this method, some accessions were recalcitrant to applied culture conditions and no or very low division frequency was noted. 'Oregon 123' and 'Badger Shipper' were classified as recalcitrant (unpublished data), becoming suitable experimental material to test the potential of PSK-α in vitro. Grzebelus et al. [32] evaluated the effect of supplementation of the culture medium with 0–0.01–0.1 and 1 µM of PSK-α in sugar beet protoplast cultures. The highest plating efficiency (20%) was observed at a concentration of 0.1 µM of PSK-α. Moreover, the same concentration of PSK-α promoted microspore embryogenesis in triticale and wheat [40]; therefore, in our study we applied this concentration of PSK-α to the culture medium.

Our results showed that the viability of protoplasts was independent from protoplast source tissue. Protoplast viability of all tested accessions on Day 1 after isolation was high (approximately 80–90%), and in that time the effect of PSK-α on protoplast viability was less obvious; however, the differences were visible later, on fifth day of culture, when viability decreased in all tested accessions. Decrease in protoplast viability over the first days of culture was reported previously [32,34] and might be associated with the damage in plasma membranes as a result of enzyme digestion and purification, osmotic stress, or oxidative stress during isolation and culture establishment [13,42]. Here, on Day 5, protoplast-derived cells of majority of tested accessions were more viable when cultured in the medium with PSK-α in comparison with the control.

In this study, mitotic activity of protoplasts was genotype-dependent, but also PSK-α supplemented to the culture medium increased mitotic activity of cultured protoplast-derived cells over twofold compared to cells cultured in the PSK-free control medium. Detailed results of the interaction of PSK-α supplementation in the culture medium and the genotype showed interesting relations – in the well-responding cultivar 'Reball F1', the effect of PSK-α was not as strong as in the low-responding breeding line LM98, where mitotic activity of protoplast-derived cells cultured in the presence of PSK was near 10-fold higher, compare to PSK-free the control. A beneficial effect of PSK-α was also observed for 'Sława z Gołębiewa', 'Ramkila F1', and breeding line LM. In recalcitrant

cultivars used in this study ('Oregon 123' and 'Badger Shipper'), although statistically insignificant, an increase in division frequencies of protoplast-derived cells cultured on PSK-α supplemented medium compared to the PSK-free control was observed.

Matsubayashi et al. [31] demonstrated the effect of phytosulfokines supplemented to the culture media on rice protoplasts. Protoplasts embedded in agarose blocks in low density cultures (5×10^4 of cells per mL of medium) and cultured in the presence of PSK-α and PSK-β divided frequently (58–66%) and gave rise to microcallus colonies after 4 weeks of cultivation. Results of experiments with mesophyll protoplast cultures of sugar beet [32] showed that plating efficiency of control cultures was 1%, while in the medium with PSK-α it increased up to 28%. The authors demonstrated that application of exogenous PSK reversed the recalcitrance of in vitro cultured mesophyll sugar beet cells. Recently, Maćkowska et al. [41] cultured protoplasts of different *Daucus* accessions in the presence of PSK. Their results showed that in five out of seven tested accessions, PSK-α at a concentration of 100 nM (0.1 μM) led to an increase in plating efficiency (55–80%) in comparison with the control (40–65%). However, for two wild *Daucus* species, stimulation of cell divisions was observed only in early cultures and callus colonies were not formed. The above-mentioned results and observed in our study differences in culture efficiency among seven tested cabbage accessions suggest that supplementation of the liquid culture media with PSK-α might increase mitotic activity of protoplast-derived cells; however, the genotype plays the main role in the responsiveness of isolated plant cells to in vitro cultures [5,7,37,43].

In protoplast cultures, cell proliferation highly depends on culture density, and it was reported that cell divisions cease in low density cultures [28,44]. Yamakawa et al. [30] reported that mitotic activity in low-density suspension cultures is not stimulated by known plant hormones or defined nutrients; however, can be induced by the addition of conditioned medium prepared from cultures of rapidly proliferating cells. The successful stimulation of cell divisions in low density cultures was induced in *B. napus* [45], but not in *B. oleracea* var. *capitata* [2,46]. In this study, we cultured protoplasts in optimal density [10] and in the presence of PSK-α; however, in two genotypes, we did not observed satisfactory stimulation of cell divisions. We used PSK-α in concentration of 0.1 μM, and it might be likely that this concentration was not efficient for these two accessions for the promotion of cell divisions. In contrast, in high-responsive genotypes PSK-α had only a minor effect.

We also observed the beneficial effect of supplementation of the protoplast culture medium in PSK on the regeneration process. Phytosulfokines have the potential to induce cell divisions, but our results showed it was also important for its maintenance. In controls without PSK-α, even if cell divisions were induced, they ceased at some point, and as a result shoot regeneration was not observed. It seems that the mitotic divisions stimulated by PSK-α were sustained even after transferring protoplast-derived callus tissue on the solid regeneration medium, what resulted in a higher percentage of regenerated shoots obtained from callus colonies developed in the media supplemented with PSK-α.

To the best of our knowledge, this is the first report showing the effect of PSK-α on *Brassica oleracea* protoplast cultures. We demonstrate that mitotic activity of cabbage protoplasts can be increased by supplementation of culture medium with PSK-α. The effect of PSK-α was more pronounced in some low-responding genotypes, and we observed 10–47% increase in division frequency of protoplast-derived cells cultured in medium supplemented with PSK-α compared to PSK-free control medium. The supplementation of the culture medium in PSK-α had a minor effect on mitotic activity of two out of seven tested accessions, showing that the genotype plays a major role in the response of cabbage to protoplast culture. PSK-α supplemented into the liquid culture medium had an indirect effect on shoot regeneration by inducing sustained cell divisions leading to an increase in shoot regeneration. Further research will be focused on the evaluation of the effect of broader spectra of PSK-α concentrations in the liquid medium for culturing cabbage protoplasts as well as on the examination of the effect of direct supplementation of solid medium with phytosulfokines on the regeneration process.

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